(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
17 October 2002 (17.10.2002)

PCT

(10) International Publication Number
WO 02/081739 A2

(51) International Patent Classification[7]: C12Q 1/68, G01N 27/49

(21) International Application Number: PCT/EP02/03892

(22) International Filing Date: 8 April 2002 (08.04.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/282,164 9 April 2001 (09.04.2001) US

(71) Applicant *(for all designated States except US)*: **FRAUNHOFER-GESELLSCHAFT ZUR FÖRDERUNG DER ANGEWANDTEN FORSCHUNG E.V.** [DE/DE]; Leonrodstrasse 54, 80636 München (DE).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **BREDEHORST, Reinhard** [DE/DE]; Eichenstrasse 55, 20255 Hamburg (DE). **HINTSCHE, Rainer** [DE/DE]; Gravensteinstrasse 61C, 13127 Berlin (DE). **HEUBERGER, Anton** [DE/DE]; Auf dem Kyberg 21, 82041 Deisenhofen (DE).

(74) Agents: **OLGEMÖLLER, Luitgard** et al.; Leonhard Olgemöller Fricke, Postfach 10 09 62, 80083 München (DE).

(81) Designated States *(national)*: JP, US.

(84) Designated States *(regional)*: European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 02/081739 A2

NON-ENZYMATIC LIPOSOME-LINKED CLOSELY SPACED ARRAY ELECTRODES ASSAY (NEL-ELA) FOR DETECTING AND QUANTIFYING NUCLEIC ACIDS

I. FIELD OF THE INVENTION

The present invention relates to non-enzymatic electrochemical assay procedures for the detection and quantification of nucleic acids and amplicons thereof using novel affinity liposomes containing electrochemically active reporter molecules.

II. BACKGROUND OF THE INVENTION

Reporter systems most frequently utilized in assays for detecting and quantifying nucleic acids in fields such as biotechnology, environmental protection, and public health include detection by fluorescence, chemiluminescence, and colorimetry. These labels have been linked or conjugated directly to nucleic acid reactants and products, or generated via nucleic acid-enzyme conjugates similar to ELISA techniques. At present, the vast majority of assays for detecting and quantifying nucleic acids is based on enzyme-linked methods in combination with optical detection systems. As a consequence, these assays depend on clean and unsoiled measurement chambers, optical clarity and compatible viscosity of sample and support media, and a neutral optical background. Miniaturization of the sample volume in the measurement chamber is limited since reliable optical determinations on the basis of Lambert-Beer's law require a reproducible length of the light path. In addition to such cumbersome requirements, optical systems are also expensive, in that they generally require a light source, transmission, photo multiplication, and signal digitalization or analog quantitation. Furthermore, conventional enzyme-linked assay procedures require generally a long analysis time due to several time-consuming washing steps and extended enzymatic incubation periods if trace amounts of analytes have to be detected. Recent advances in template amplification and signal amplification methods in conjunction with enzymatic detection procedures with colorimetric or chemiluminescent substrates have resulted in highly sensitive nucleic acid detection and quantitation techniques, but they rely still on enzyme-mediated signal amplification techniques and optical detection methods which suffer from the above listed drawbacks.

Attempts have been made to provide detection systems other than optical ones and to establish assay procedures requiring less analysis time. An immunoassay for digoxin using p-aminophenol as electrochemically detectable reporter molecule

has been described by Hang, H.T., et al., Anal. Chim. Acta 214, 187, 1988. The assay sensitivity, however, is very low and suffers from the time-consuming drawbacks of enzyme-linked detection methods. Separation-free electrochemical detection procedures have been described recently by Duan, C., and Meyerhoff, M.E. (Anal. Chem. 66, 1369, 1994) and Meyerhoff, M.E., Duan, C., and Meusel, M. (Clin. Chem. 41, 1378, 1995). Their systems avoid some of the disadvantages associated with optical ELISA-type assays, but the detection sensitivity is still too low despite an enzymatic amplification step. An improved method for small-volume voltammetric detection of p-aminophenol by an interdigitated array microelectrode cell has been reported by Niwa, O., et al., Anal. Chem. 65, 1559, 1993. However, using that type of interdigitated array electrodes and alkaline phosphatase for amplification, the detection sensitivity of IgG antibodies was still in a range that is not viable for clinical applications. An electrochemical amplification step has been realized by redox recycling of electrochemically redox active reporter molecules such as p-aminophenol between the adjacent microband electrodes of an interdigitated electrode array, with each electrode within the diffusion layer of the other. Redox recycling occurs when both the oxidation and the reduction potential of the reversible redox species are applied to pairs of interdigitated electrodes. Using the redox recycling procedure and interdigitated electrodes with an electrode width of 1.5 μm and an interelectrode spacing of 0.8 μm, the detection limit of several redox mediators including p-aminophenol, o-hydroxychinone, o-benzoquinone, and ferrocene lysine could be lowered to 5 – 10 nmol/l (Hintsche, R., Paeschke, M., Uhlig, A., and Seitz, R. In: Frontiers in Biosensorics. Fundamental Aspects (F.W. Scheller, F. Schubert, J. Fedrowitz, eds.), pp. 267-283, Birkhauser Verlag, Basel, Switzerland, 1997). However, none of the above listed techniques teaches how to design an assay procedure for detecting and quantifying nucleic acids. Furthermore, the detection limit of 5 – 10 nmol/l achieved by Hintsche and coworkers via redox recycling in closely spaced interdigitated array microelectrodes requires the use of enzymatic amplification systems which are relatively expensive. Since only a limited number of enzyme molecules can be attached to each detector molecule, highly purified enzyme preparations are required for the synthesis of enzyme-detector molecule conjugates to avoid coupling of enzymatically inactive contaminating proteins to detector molecules. Highly purified enzymes, however, are expensive due to several labor-intensive purification steps.

The application of array microelectrodes for the selective detection of redox species in the presence of interfering molecules has been described by several investigators (e.g. Sanderson, D.G., and Anderson, L.B. Anal. Chem. 57, 2388, 1985; Niwa, O., et al., W.R. Anal. Chem. 65, 1559, 1993). For these analyses, however, interdigitated arrays with relatively large electrodes spaced apart from each other by several micrometer were used.

The use of enzymatic amplification systems for electrochemical detection procedures poses additional problems in that the reporter molecules have to be chemically modified to generate substrates which are electrochemically inactive prior to enzymatic action. For example, p-aminophenol has been adjusted for the alkaline phosphatase amplification system by converting the reporter molecule to the electrochemically inactive p-aminophenylphosphate. For many other reporter molecules such as osmium- or ruthenium-containing redox mediators, however, a similar adjustment has not been reported and is likely to pose substantial synthetic hurdles. On the other hand, osmium- as well as ruthenium-containing redox mediators would be very useful for electrochemical detection systems since they provide a combination of important advantages for redox recycling including a reversible redox behaviour at electrodes, low oxidation/reduction potentials, slow oxidation by oxygen, and an excellent solubility in aqueous media.

Alternatively, reporter molecules may be immobilized onto solid support matrices via enzymatically cleavable spacer compounds. This approach allows immobilized redox mediators to remain electrochemically active since access to the electrodes requires enzymatic cleavage of the spacer compound. The technology, however, requires additional solid support systems and is likely to provide only moderate amplification due to the limited amount of redox mediators that can be immobilized onto solid supports and the unfavorable enzyme kinetics at solid-liquid interfaces.

Recently, Le Gal La Salle, A., et al. (Anal. Chem. 34, 1245, 1995) have developed an approach that amplifies the detection of an electrochemically active enzyme product and concomitantly discriminates the substrate signal, although it possesses the same oxidation potential as the product. N-Ferrocenoyl-6-amino-2,4-dimethylphenyl phosphate disodium salt is used as substrate for the alkaline phosphatase amplification system and the corresponding phenol product is entrapped selectively and irreversibly in a Nafion film electrode as a ferricinium salt by applying an anodic potential. Despite its high detection sensitivity, this

method has the limitation that the Nafion film electrode can be used only once since the product is strongly retained within the polymer and phenyl derivative radicals are electropolymerized during the course of the anodic preconcentration. In an attempt to overcome this limitation, the sodium salt of ferrocene ethyl phosphate ester $(FcEtOPO_3)^{2-}$ has been synthesized as a new alkaline phosphatase substrate (Limoges, B., and Degrand, C. Anal. Chem. 68, 4141, 1996). The enzyme product, ferrocene ethanol, is less hydrophobic than N-ferrocenoyl-6-amino-2,4-dimethylphenol and can be released from the Nafion film in its neutral form by cathodic stripping. The limited efficiency of the cathodic stripping procedure, however, restricts the reuse of the same electrode to a small number of analyses which is not in accordance with the requirements of cost effective assay procedures.

A non-enzymatic electrochemical assay for the detection of triazine pesticides has been described by Bäumner and Schmid (Biosensors Bioelectronics 13, 519, 1998). In this immunomigration assay procedure, immobilized monoclonal antibodies against atrazine and terbutylazine serve as biorecognition element and ascorbic acid encapsulated in hapten-tagged liposomes is used as reporter system for amperometric detection by thick-film electrodes printed on PVC. The assay principle is based on a competitive binding reaction of triazine pesticides and hapten-tagged liposomes to the immobilized monoclonal antibodies, followed by immunomigration of nonbound liposomes to a zone containing detergent at a concentration sufficient to release the liposome-entrapped ascobic acid. The method is capable of detecting triazine pesticides in tap water at a concentration of approximately 1 μg/liter. The technique described in this reference, however, is neither applicable for detecting and quantifying nucleic acids nor does it teach how to design such an assay procedure.

Liposomes as carriers of entrapped reporter molecules have also been employed in many other non-enzymatic assay procedures. The most frequently performed non-enzymatic liposome-based assays utilize liposomes that contain encapsulated fluorophores and surface-attached antigens or antibodies for the determination of proteinaceous antigens or antibodies in homogeneous or heterogeneous immunoassays (Diamandis, E.P., and Christopoulos, T.K. (eds.) Immunoassay, Academic Press, San Diego, p. 322 (1996). However, such assay procedures termed liposome immunosorbent assays (LISA), are also not suitable

to detect and quantify nucleic acids via non-enzymatic electrochemical liposome-based techniques.

The problems listed in the foregoing describe many of the factors that limit the potential value of existing non-enzymatic assay procedures for electrochemical detection and quantification nucleic acids. It is obvious that there exists a need in the field for an electrochemical assay suitable for detecting and quantifying nucleic acids by an enzyme-independent amplification technique that provides a cost effective method for fast analyses with a satisfactory level of detection sensitivity.

III. SUMMARY OF THE INVENTION

It is the object of the present invention to provide a novel reporter system for the detection and quantification of nucleic acids and amplicons thereof which substantially overcomes the limitations known in the prior art.

In a first aspect of the invention, a reporter system for detecting an analyte, selected from nucleic acids and their amplicons, in a solution is provided, comprising the following components: (i) a solid support containing immobilized thereon capture oligonucleotides capable of binding to said analyte, preferably by forming specific helical complexes therewith, (ii) affinity liposomes containing encapsulated electrochemically detectable reporter molecules, preferably redox mediators, and surface-attached affinity components capable of specifically binding to said analyte and/or said capture oligonucleotide in a condition where analyte and capture oligonucleotides are bound to each other, but not to free capture oligonucleotides, and (iii) an electrochemical sensor for voltammetric, e.g. amperometric detection of electrochemical detectable reporter molecules.

In a second aspect of the invention, a method for detecting and optionally quantifying an analyte, selected from nucleic acids and their amplicons, in a liquid sample to be analyzed is provided, comprising the steps of (a) providing (i) a solid support containing immobilized thereon capture oligonucleotides capable of binding said analyte, preferably by forming specific helical complexes therewith, and (ii) an electrochemical sensor, (b) contacting the sample to be analyzed with the immobilized capture oligonucleotides, (c) adding affinity liposomes or systems comprising affinity liposomes which contain encapsulated electrochemically detectable reporter molecules, preferably redox mediators, the affinity liposomes or systems comprising further surface-attached affinity components capable of

specifically binding to the analyte to be detected and/or the said capture oligonucleotides in a condition where analyte and capture oligonucleotides are bound to each other, but not not free capture oligonucleotides, (d) removing unbound affinity liposomes, (e) releasing encapsulated electrochemically detectable reporter molecules from the interior of affinity liposomes bound to analyte and/or capture oligonucleotides as defined above, and (e) detecting reporter molecules thereby released using the electrochemical sensor.

Preferably, the electrochemical sensor is a closely spaced array (micrometer or submicrometer scale) of electrodes for voltammetric, e.g. and preferably amperometric detection of electrochemically detectable reporter molecules, the electrodes consisting e.g. of thin film nobel metal or carbon.

The components of this invention allow the design of various electrochemical assay configurations. Preferred assay procedures include binding of affinity liposomes to captured nucleic acids and amplicons thereof, release of redox mediators from specifically bound affinity liposomes, and voltammetric quantification of released redox mediators via redox recycling.

Depending on the lipid composition of affinity liposomes, the release of encapsulated redox mediators from specifically bound liposomes may be effected e.g. by detergent-mediated lysis or by a moderate increase of the ambient temperature. The quantity of redox mediators is a proportional measure of the quantity of target nucleic acid or amplicons thereof in the specimen.

In a specific and preferred embodiment of the invention, polymeric carrier molecules capable of binding multiple affinity liposomes and/or preformed complexes of affinity liposomes are utilized for amplified assay procedures.

IV. BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the general assay procedure using affinity liposomes having surface-attached intercalating agents which serve as affinity components capable of specifically binding to the analyte in a condition where analyte and capture oligonucleotides are bound to each other as defined above. Figure legend: solid support (1); surface bound capture oligonucleotide (2); target DNA (3); region of DNA double helix (4);

affinity liposome (5); intercalating residues (In) (6); flexible spacer molecules (7); redox mediators (8).

Figure 2 shows the general assay procedure using affinity liposomes having surface-attached oligonucleotides which serve as affinity components capable of specifically binding to the analyte in a condition where the said analyte is bound to the capture oligonucleotides as defined above . Figure legend: solid support (1); surface bound capture oligonucleotide (2); target DNA (3); region of DNA double helix (4); affinity liposome (5); flexible spacer molecules (7); redox mediators (8); single-stranded oligonucleotide (9).

Figure 3 shows an assay procedure comparable to that of figure 1, but using additional polymeric carrier moecules for signal amplification. Figure legend: solid support (1); surface bound capture oligonucleotide (2); target DNA (3); region of DNA double helix (4); affinity liposome (5); intercalating residues (In) (6); flexible spacer molecules (7); redox mediators (8); polymeric carrier molecule (10); hapten (11); anti-hapten antibody (12).

V. DETAILED DESCRIPTION OF THE INVENTION

In its broadest aspect, the novel electrochemical reporter technology of this invention is capable of detecting and quantifying target nucleic acids or their amplicons by molecular biological and immunochemical procedures for analytical and clinical applications. Analyses of specific nucleic acids or nucleic acid sequences are commonly, but not exclusively used to examine body fluids or tissues for the presence of infectious microorganisms, malignancies, inherited genetic defects, pharmacogenomics, forensic medical evidence, and paternity / maternity identification.

The present invention employs a solid-support with immobilized capture oligonucleotides, affinity liposomes, and an electrochemical sensor. Capture oligonucleotides are selected from a group including single-chain nucleic acids (ribo and deoxyribo nucleic acids), single-chain oligonucleotides (ribo and deoxyribo oligonucleotides), and 'preorganized' oligonucleotide structures including peptide nucleic acid (PNA) analogues. 'Preorganized' oligonucleotide structures offer a potential advantage since they exhibit a higher affinity for target nucleic acids provided they are rigidified prior to binding in a position that resembles the bound conformation.

The affinity liposomes of the present invention contain encapsulated electrochemically detectable reporter molecules and surface-attached affinity components capable of specifically binding to the analyte to be detected and/or to the said oligonucleotide capture oligonucleotides in a condition where analyte and capture oligonucleotides are bound to each other, but not not free captureoligonucleotides. Respective affinity components include but are not limited to single-chain nucleic acids (ribo and deoxyribo nucleic acids), single-chain oligonucleotides (ribo and deoxyribo oligonucleotides), 'preorganized' oligonucleotide structures including peptide nucleic acid (PNA) analogues, intercalating agents, intercalating agents conjugated to oligonucleotides or nucleic acids, immunoglobulins or fragments of immunoglobulins with specificity for double- and/or triple-stranded nucleic acids), and nucleic acid binding proteins (e.g., the prokaryotic lac repressor or the eukaryotic cyclic AMP responsive element binding protein (CREB)). The nucleic acids and oligonucleotides are designed for hybridization to single-stranded segments of captured target nucleic acids or amplicons thereof, while binding to the capture oligonucleotides should be substantially not possible. Suitable intercalating agents are selected from a group exhibiting a preference for double-stranded and/or triple-stranded nucleic acids including actinomycin D derivatives, anthracycline derivatives, acridine derivatives, cyanine dye derivatives, hydroxystilbamidine derivatives, imidazole derivatives, indole derivatives, phenanthridine derivatives, and psoralen derivatives.

Useful as encapsulated electrochemically detectable reporter molecules are those which can undergo oxidation or reduction e.g. hydrochinones, naphthols, or organimetals like complexes of Os, Ru or Co or the like. Preferably, the reperter molecules are capable to undergo redox recycling. Such molecules may be

aromatic redox mediators or organic and anorganic metal complexes containing osmium, ruthenium, iron, copper, and chromium. Examples of suitable aromatic redox mediators include p-aminophenol and derivatives thereof, catechol and derivatives thereof, dopamine and derivatives thereof, methoxytyraminin and derivatives thereof, aromatic compounds with more than one aromatic ring structure such as anthracene derivatives, and heterocyclic aromatic compounds such as serotonin, hydroxyindol acetic acid, and derivatives thereof. Examples of metal complexes contain the metal complexed by various aromatic and heterocyclic aromatic compounds, the side chains of which are derivatized with residues such as carboxyl groups, halogens, aminoethyl groups, and pyridine derivatives for adjustment of the redox potential according to the specific assay requirements.

The release of electrochemically detectable reporter molecules encapsulated in specifically bound affinity liposomes is effected e.g. by an increase of the ambient temperature (Method A) or the addition of liposome-lysing solvents such as organic solvents or detergents (Method B).

Detection and quantification with an electrochemical sensor preferably comprises a closely spaced array (micrometer and submicrometer scale), the electrodes for example consisting of thin film nobel metal. Preferred are interdigitated arrays where anodes and cathodes have a width between 10 and about 5000 nm and the electrodes are spaced apart from each other with a distance between 5 and 5000 nm. More preferred are interdigitated arrays where the electrodes are spaced apart from each other with a distance between 100 and about 800 nm.

In one preferred embodiment of the present invention, closely spaced array microelectrodes are utilized where the electrodes are spaced apart from each other with a distance of 10 to 1000 nm, more preferred of 100 to 800 nm. Since the mass transfer of the redox reporter molecules between anodes and cathodes is accomplished by diffusion only, a narrow interelectrode gap reduces the diffusion length and, thereby, increases the efficiency of recycling processes, minimizes intramolecular cyclization of redox reporter molecules and potential electron transfer processes as described for oxidized catecholamines and L-ascorbic acid (Niwa o., Morita, M., and Tabei, H. Electroanalysis 3, 163, 1991).

An electrochemical sensor comprised of an interdigitated array of microelectrodes of the kind useful in the pactice of the present invention is published in

International Patent Application (Hintsche, R., et al., PCT Publication No. WO 94/29708). The publication discloses an array consisting of four pairs of comb-shaped interdigitated anodes and cathodes parallel arranged on a planar silicon chip. Conductors connecting the electrodes to electrical contact surfaces are covered by an insulating layer.

The basic assay procedure includes binding of the analyte to immobilized capture oligonucleotides by molecular biological interactions, detection of captured analyte by analyte-specific binding of affinity liposomes containing encapsulated electrochemically detectable reporter molecules, removal of non-bound affinity liposomes, release of electrochemically detectable reporter molecules from the said specifically bound liposomes, and detection and optionally quantification of such reporter molecules by means of voltammetry or amperometry, preferably using interdigitated array electrodes (Figures 1 and 2). The quantity of released electrochemically detectable reporter molecules is a proportional measure of the amount of target nucleic acids (analyte) in the specimen. The expression "analyte-specific binding of affinity liposomes" does not only mean binding of affinity liposomes to analyte bound to capture oligonucleotides , but also includes binding of affinity liposomes to structures of the capture oligonucleotides which are affected by the binding event with the analyte or to structures of the capture oligonucleotide-analyte combination ("complex") thus formed (e.g. double stranded nucleic acid formed upon adding single stranded nucleic acid analyte to a capture oligonucleotide comprising single stranded nucleic acid) and binding to polymeric carriers which in turn are bound to affected capture oligonucleotide and/or bound analyte.

Reporter systems and assay procedures including additional or modified steps are specific embodiments of the basic concept illustrated above.

In one preferred embodiment, additional amplification molecules are included in the assay procedure for signal amplification. In one amplified assay procedure, captured analyte (target nucleic acids or amplicons thereof) is detected by polymeric carrier molecules containing two different affinity components, one for specific binding to captured nucleic acids and/or to capture oligonucleotides in a condition where analyte and capture oligonucleotides are bound to each other, but not to free capture oligonucleotide (e.g., intercalating agents, oligonucleotides, or antibodies with specificity for double- and/or triple-stranded nucleic acids) and the other for binding of affinity liposomes. Preferred affinity components for binding of

affinity liposomes to polymeric carrier molecules are hapten / anti-hapten antibody systems, enzyme inhibitor / enzyme systems, and the biotin / (strept)avidin system. Since polymers allow covalent attachment of multiple affinity components, each captured nucleic acid molecule binds a multitude of affinity liposomes leading to an effective signal amplification. Preferred examples of synthetic and natural polymer derivatives include, but are not limited to derivatives of polysaccharides, polyamino acids, polyvinyl alcohols, polyvinylpyrrolidinones, polyacrylic acids, various polyurethanes, polyphosphazenes, and copolymers of such polymers. In a more preferred embodiment, derivatives of dextran are employed as polymeric carrier molecules.

In one embodiment of the present invention using such polymeric carrier molecules, captured analyte (i.e., captured target nucleic acids or amplicons thereof) is detected by dextran polymers containing two types of low molecular weight affinity components. One of the covalently linked affinity components is capable of specifically binding to the captured analyte in a structure restricted manner (e.g. intercalating agents or oligonucleotides). The other covalently linked affinity component (e.g., hapten molecules, enzyme inhibitors, or biotin residues) ise capable of specifically binding multiple affinity liposomes. Preferably, more than one of the other such component is linked to the polymeric carrier molecule. Subsequently, bound dextran polymers are detected by affinity liposomes containing surface-attached proteinaceous affinity components (e.g., anti-hapten antibodies, enzyme molecules, or (strept)avidin) capable of binding to the affinity components on the dextran polymers (Fig. 3). Quantitation of electrochemically detectable reporter molecules encapsulated in specifically bound affinity liposomes is performed as described.

In another embodiment of the present invention utilizing such polymeric carrier molecules, analyte (captured target nucleic acids or amplicons thereof) is detected by dextran polymers containing two types of proteinaceous affinity components. One of the covalently linked affinity components is capable of specifically binding to a specific captured target nucleic acid or its amplicons in a structure restricted manner (e.g., antibodies with specificity for double- and/or triple-stranded nucleic acids). The other covalently linked affinity components (e.g., anti-hapten antibodies, enzyme molecules, or (strept)avidin) are capable of specifically binding multiple affinity liposomes. Subsequently, bound dextran polymers are detected by affinity liposomes containing surface-attached low molecular weight affinity components (e.g., hapten molecules, enzyme inhibitors, or biotin residues)

capable of binding to the proteinaceous affinity components on the dextran polymers (Fig. 3). Quantitation of electrochemically detectable reporter molecules encapsulated in specifically bound affinity liposomes is performed as described.

In a third embodiment of the present invention utilizing such polymeric carrier molecules, captured target nucleic acids or amplicons thereof are detected by dextran polymers containing proteinaceous affinity components derivatized with low molecular weight affinity components. If the proteinaceous affinity component is capable of specifically binding to a specific captured target nucleic acid or its amplicons in a structure restricted manner (e.g., antibodies with specificity for double- and/or triple-stranded nucleic acids), the low molecular weight affinity components (e.g., hapten molecules, enzyme inhibitors, or biotin residues) are capable of specifically binding affinity liposomes containing surface-attached proteinaceous affinity components (e.g., anti-hapten antibodies, enzyme molecules, or (strept)avidin) capable of binding to the low molecular weight affinity components on the dextran polymers. Vice versa, if the low molecular weight affinity components (e.g., intercalating agents or oligonucleotides) are capable of specifically binding to a specific captured target nucleic acid or its amplicons in a structure restricted manner, the proteinaceous affinity components (e.g., anti-hapten antibodies, enzyme molecules, or (strept)avidin) are capable of specifically binding affinity liposomes containing surface-attached low molecular weight affinity components (e.g., hapten molecules, enzyme inhibitors, or biotin residues) capable of binding to the proteinaceous affinity components on the dextran polymers. Quantitation of electrochemically detectable reporter molecules encapsulated in specifically bound affinity liposomes is performed as described.

In another preferred embodiment of the present invention, signal amplification is achieved by using preformed complexes of affinity liposomes. One preferred method for the preparation of complexed affinity liposomes utilizes affinity liposomes containing two types of surface-attached affinity components, one for specific binding to captured nucleic acids or amplicons thereof in a structure restricted manner (e.g., intercalating agents or oligonucleotides) and the other for complexation of affinity liposomes via bridging molecules. Suitable bridging molecules are selected from a group including a) bi- or oligovalent anti-hapten antibodies or fragments thereof, as well as conjugates or fusion constructs thereof; b) enzymes, enzyme conjugates, and fusion constructs of enzymes providing more than one inhibitor-binding site; and c) avidin and streptavidin. Any bridging molecule that provides more than one binding site is useful for this type of amplification methodology.

A first group of preformed complexes of affinity liposomes useful for this invention include complexes generated by reaction of i) (strept)avidin with affinity liposomes containing surface-attached biotin residues and surface-attached nucleic acid-reactive components (e.g., intercalating agents or oligonucleotides), ii) anti-hapten antibody molecules providing more than one hapten-binding site with affinity liposomes containing surface-attached hapten molecules and surface-attached nucleic acid-reactive components (e.g., intercalating agents or oligonucleotides), and iii) enzyme conjugates providing more than one inhibitor-binding site with affinity liposomes containing surface-attached inhibitor molecules and surface-attached nucleic acid-reactive components (e.g., intercalating agents or oligonucleotides).

In another embodiment of the invention, polymeric carrier molecules containing covalently coupled affinity components are employed as bridging molecules for the preparation of preformed complexes of affinity liposomes. The application of polymeric carrier molecules for complexation of affinity liposomes is useful when affinity components are utilized as bridging molecules which contain only a single binding site for the correponding affinity partner (e.g., anti-hapten single-chain antibodies (scFv) and enzymes containing only a single inhibitor binding site).

In still another embodiment, complexed affinity liposomes are employed which are prepared utilizing two types (type I and type II) of affinity liposomes containing different affinity components. The affinity components on type I affinity liposomes are capable of specifically binding to specific captured nucleic acids or amplicons thereof (e.g., single-stranded oligonucleotides complementary to single-stranded segments of captured target nucleic acid or amplicons thereof) and/or to capture oligonucleotides in a condition where analyte and capture oligonucleotides are bound to each other, but not to free capture oligonucleotide. The affinity components on type II affinity liposomes are capable of specifically binding to the affinity components on type I affinity liposomes (e.g., single-stranded oligonucleotides complementary to type I single-stranded oligonucleotides).

In the method for detecting an analyte using such preformed complexes of affinity liposomes, the analyte to be detected is bound to immobilized capture oligonucleotides by molecular biological interactions and detected by specific binding of said preformed complexes of affinity liposomes containing surface-attached nucleic acid-reactive affinity components (Fig. 4).

In another embodiment of the amplification assay procedure, signal amplification is achieved by a combination of polymeric carrier systems and preformed complexes of affinity liposomes. One example for such a combination uses dextran polymers containing two types of covalently linked affinity components, one being capable of specifically binding to the captured target nucleic acid or its amplicons to be detected in a structure restricted manner, and the other being capable of specifically binding preformed complexes of affinity liposomes.

Thus, the present invention provides a detection system that eliminates i) the necessity for optical clarity of the sample solution and other ambient optical density requirements, and ii) the necessity of enzymatic amplification technology. One small affinity liposome provides up to 10^5 molecules of redox mediators to allow excellent detectability of a binding event. Using a silion microchip-formatted array of closely spaced (micrometer and submicrometer scale) thin fim nobel metal electrodes for quantitation of liposome-encapsulated redox mediators, a volume of only a few microliters is required for voltammetric or amperometric detection. Consequently, the liposome-encapsulated redox mediators can be released into a very small volume, thereby generating a relatively high concentration of released redox mediators. For example, the release of 10^5 molecules of redox mediator from a single affinity liposome into a detection volume of 2 µl would create a redox mediator concentration of approximately 0.1 pM. The signal provided by a single analyte-bound affinity liposome can be easily amplified by one to two orders of magnitude if polymeric carrier systems and/or preformed complexes of affinity liposomes are used for detection. Furthermore, the liposomal encapsulation technique allows the use of a large variety of redox mediators including those which provide highly efficient redox recycling, but are difficult to chemically adjust for enzyme amplification systems. There is no need for chemical derivatization of redox mediators using the non-enzymatic liposome-linked interdigital array electrodes assay. As a result, the present invention reduces costs and analysis time without compromising detection sensitivity.

The reporter system as described above may be provided in the form of a kit, the components of which are (i) a solid support containing the said immobilized capture oligonucleotides, (ii) the affinity liposomes containing at least one surface-attached affinity component, which comprise the said electrochemically detectable reporter molecules, and (iii) the said electrochemical sensor ("transducer").

A specific arrangement is not required because measurement of the reporter molecules is independent of the immediate presence of the capture oligonucleotides. It is one of the advantages of the present invention that contacting the sample to be measured for the presence of analyte may be performed independent of performing the other steps of the inventive method, as time and/or area of performing the single method steps are concerned, provided that the components are sufficiently stable. Thus, measurement of the reporter molecules may be performed in/on the same or in another vessel or plate (e.g. microtiter plate) in which the analyte had been bound to the capture oligonucleotides, and also possibly within a time limit which is suitably selected. It is possible that the electrochemical sensor is on the same support which carries the immobilized capture oligonucleotides, but this is not a necessary requirement. In one embodiment of the invention, the capture oligonucletides may be immobilized in the space between single electrodes. Alternatively, the support carrying the immobilized capture oligonucleotides is provided on a flat substrate which may be inserted into the container in which the electrochemical detection is intended to be made. In a preferred embodiment, the solid support containing immobilized capture oligonucleotides is at least a part or structure of the container or vessel In which the detection or a part thereof shall be performed. For example, the solid support is part of a variety of wells in a plate (e.g. microtiter plate), or is part of the bottom or the walls of the container or microtiter plate. In another embodiment of the invention, the support carrying the immobilized capture oligonucleotides is provided having the form of beads or a stripe or bar which is added to the sample which possibly contains the analyte.

If the support carrying the immobilized capture oligonucleotides is not part of the container, it may be added before or after the addition of the affinity liposomes to the sample in which the analyte is to be detected. Care should be taken that the affinity liposomes bound to capture oligonucleotides remain wet or submersed in the liquid als long as they shall be kept intact. In this way, release of reporter molecules therefrom may be deferred. Hence, the method of the present invention results in the release of reporter molecules caused by the presence of analyte to be detected, while detection of the said reporter molecules may be performed separately (in regard to place and time). Alternatively, all steps to be performed in the present method may be performed in only one vessel and/or in an immediate sequence.

Subsequently, the components of the present invention are described in more detail.

V.1. CAPTURE OLIGONUCLEOTIDES

As mentioned before, the invention comprises a solid support with immobilized capture oligonucleotides for specific binding of target nucleic acids. Capture oligonucleotides may be selected e.g. from single-chain nucleic acids (ribo and deoxyribo nucleic acids) and single-chain oligonucleotides (ribo and deoxyribo oligonucleotides). The oligonucleotides are designed to form specific helical complexes with target nucleic acids. In a more preferred embodiment, 'preorganized' oligonucleotide structures are used as capture oligonucleotides (for a review, see Kool, E.T., Chem. Rev. 97, 1473, 1997). The rational for using 'preorganized' oligonucleotide structures is based on the observation that the affinity of oligonucleotides for the target nucleic acid can be increased by modifications rigidifying the oligonucleotide prior to binding so that it more resembles the bound conformation. An oligonucleotide rigidly held in the binding position prior to complexation shows less free internal bond rotations that need to be 'frozen' during complexation. At the same time, the molecule is organized into a shape which is more complementary to the desired target than to undesired ones. This increases selectivity because mismatched targets will cause unfavorable responses such as non-optimum bond angles or streric clashes.

Strategies for the construction of 'preorganized' oligonucleotide structures include i) enhancement of base stacking, ii) limitation of bond rotations, and iii) linking of binding domains. Double-, triple-, and quadruple-stranded nucleic acid helices are stabilized by base stacking and hydrogen bonding interactions. Since the majority of the base-stacking interaction in nucleic acids is between bases within a strand, the strengthening of stacking will have the tendency to cause single-stranded oligonucleotides to become preorganized into a more regular helical conformation. This will therefore favor complexation by lowering the entropic cost. Examples for strategies for increasing stacking are the addition of simple substituents to DNA bases (e.g., methylation of pyrimidines at C-5), an increase of the surface area of DNA bases (e.g., by addition of aromatic heterocyclic groups to the C-5 position of pyrimidines), and the use of nonpolar DNA base analogues.

Examples for strategies for limiting bond rotations prior to complexation employ covalent bonds and include the synthesis of i) backbones with restricted freedom, ii) bicyclo-DNA, iii) hexose-DNA, and iiii) circular DNA. Prefered examples of

nucleic acid derivatives with rigid backbones include peptide nucleic acid (PNA) analogues containing amide bonds. Since an amide has restricted rotation about the carbonyl-nitrogen bond, PNAs are capable of forming strong duplexes with DNA at lowered ionic strength and very strong triplexes even at normal ionic strength. In the bicyclo-DNA approach, the normally flexible furanose ring is rigidified by addition of an ethylene bridge from C-3' to C-5', thereby forming a second five-membered ring to the natural structure. Since five-membered rings are considerably more flexible than six-membered rings, DNA analogues have been synthesized in which the furanose ring is expanded to a six-membered ring. In some cases, such oligonucleotide analogues hybridized more strongly to DNA than the natural furanose-based structures. Another preferred way to significantly limit the conformational freedom of a flexible oligonucleotide is to cyclize the chain. A circular oligonucleotide can bind a single-stranded target RNA or DNA by forming standard Watson-Crick bonds. However, such binding is limited because of the helical twist of DNA.

It is possible to use the linking of binding domains as strategy for preorganization. One aspect of critical importance for this strategy is the design of the linking group or groups. For optimal preorganization and thus highest affinity and selectivity, a linker should be both rigid and orient the binding domains in the productive geometry. Noncovalent links between binding domains have the advantage of simplifying the synthesis, but have the disadvantage of being relatively weak, thereby limiting effective preorganization. Preferred are covalent links between binding domains. For example, thiols may be placed into opposite strands of a duplex-forming sequence and used for disulfide cross-linking upon oxidation. Such duplexes become stabilized thermodynamically, presumably beause of the entropic benefit.

Using covalent links between domains, preorganized oligonucleotide structures can be prepared that lead to triplex formation on single-stranded targets. Triple-helical nucleic acid structures are known since 1957. A purine DNA base can form hydrogen-bonded contacts on two sides, one termed the Watson-Crick face and the other Hoogsteen face. Thus, in duplex DNA, a purine stretch presents sites in the major groove for Hoogsteen complexation by a third strand. Single-stranded DNA can also serve as a target for triple helix formation, since a purine stretch can be bound on two sides by a molecule carrying both a Watson-Crick complementary domain and a Hoogsteen complementary domain. A second motif for triplex formation is the so-called purine motif, in which purine-purine-pyrimidine

base triads are formed. As with the previous motif, a purine strand is in the middle, with two other strands forming hydrogen-bonded contacts. In such a motif, the Watson-Crick complementary pyrimidine strand represents the target and the other two strands the binding domains which are preorganized by linking.

A simple way to link two such triplex-forming domains is to connect them with extra non-pairing nucleotides or by a non-nucleotide linker such as hexaethylene glycol (clamp or fold-back oligonucleotides). A preferred strategy is to link two triplex-forming domains by two loops at both ends using nucleotide loops or non-nucleotide linkers (circular and looped oligonucleotides). Clamp-like oligonucleotides have been shown to bind target sequences with an 11°C advantage in T_m, whereas closure of the clamp into a full circle gave a 19°C advantage.

The clamp and circular oligonucleotide approaches are strategies in which two DNA-binding domains are linked at their end or ends. Another preferred approach is to link them across the center, thereby generating molecules with an 'H'-form. Examination of the base triads involved in triple helix shows that a bridge can easily link two C-5 positions on pyrimidines in opposite strands. Experimental results have shown that such a molecule cross-linked by a disulfide bridge (via thiopropyne-substituted thymidine nucleosides) binds a target strand more strongly than a clamp-like oligonucleotide (Chaudhuri, N.C., and Kool, E.T. J. Am. Chem Soc. 117, 10434, 1995). In a more preferred embodiment, the same strategy is used to cross-link a circular oligonucleotide with a disulfide bridge across the center. Thermal denaturation studies have shown that such bicyclic oligonucleotides bind complementary DNA strands with extremely high affinity (see Chaudhuri et al., above). Sequence selectivity was also found to benefit from this additional preorganization strategy.

Alternatively, it is possible to link together multiple binding domains (tethered DNA). For maximum cooperativity, a rigid linking domain is preferred. Although flexible linkers may not maximize affinity and selectivity, they may provide utility in some cases. For example, flexible tethers may be used to link two DNA-binding sequences for hybridization to separate sites such as purine stretches separated by non-homopurine segments.

V.2. IMMOBILIZATION OF CAPTURE OLIGONUCLEOTIDES

The solid support serving for immobilization of capture oligonucleotides may be the same support which carries the electrochemical sensor or may be a different one. The support may have e.g. a flat structure, or it may have the form of beads or the like, e.g. glassy, polymeric, or magnetic beads. The support may be part of microtiter plates used for the detection of the analyte, or be a porous, impervious, fibrous, or metallic matrix or membrane or the like, as is well-known in the art. Such matrices may be utilized as derivatized or non-derivatized solid supports for immobilization of capture oligonucleotides. Immobilization of capture oligonucleotides or derivatives thereof may be accomplished non-covalently or covalently by any of the well-known chemical coupling methods. For most applications, covalent immobilization techniques are preferred. Electrically mediated coupling procedures are equally applicable for use with the current invention. Also applicable for this invention are non-covalent, non-adsorptive immobilization techniques. For example, capture oligonucleotides derivatized at their termini with biotin residues may be immobilized onto solid supports functionalized by adsorption or covalent binding of streptavidin beforehand. The methods by which capture oligonucleotides may be derivatized with reactive residues for immobilization onto solid supports are numerous. Preferred methods include but are not limited to those which allow selective derivatization of the termini to guarantee efficient hybridization with target nucleic acids.

V.2.1. Chemical modification of the terminal 5'-phosphate group of capture oligonucleotides

In one embodiment, capture oligonucleotides containing a 5'-phosphate group are used which are derivatized with amine or sulfhydryl terminal spacer molecules for immobilization onto amine-reactive or sulfhydryl-reactive solid supports (Hermanson, G.T. Bioconjugate techniques, Academic Press, San Diego, 1996). For example, the 5'-phosphate groups of capture oligonucleotides may be reacted with carbodiimide in the presence of imidazole to form active phosphorimidazolide intermediates. These derivatives are highly reactive with diamines or bis-hydrazide compounds, forming amine terminal spacer molecules via phosphoramidate linkages. Derivatization of the 5'-phosphate groups with cystamine creates an amine terminal spacer containing a disulfide group. Reduction of the cystamine-labeled oligonucleotide using a disulfide reducing agent releases 2-mercaptoethylamine and generates a terminal thiol group.

In another preferred embodiment, sulfhydryl groups are introduced at the 5'-termini of oligonucleotides by automated solid-phase synthesis using S-

triphenylmethyl O-methoxymorpholinophosphite derivatives of 2-mercaptoethanol, 3-mercaptopropan (1) ol, or 6-mercaptohexan (1) ol (Connolly, B.A., and Rider, P., Nucleic Acids Res. 13, 4485, 1985). After clevage from the resin and removal of the phosphate and base protecting groups, oligonucleotides are obtained which contain an S-triphenylmethyl group attached to the 5'-phosphate group via a two, three, or six carbon chain. The triphenylmethyl group can readily be removed with silver nitrate to give the free thiol. Alternatively, primary amino groups can be introduced at the 5'-termini of oligonucleotides by automated solid-phase synthesis using N-monomethoxytrityl-O-methoxydiisopropylaminophosphinyl 3-aminopropan (1) ol (Connolly, B.A. Nucleic Acids Res. 15, 3131, 1987). After cleavage from the resin and removal of the phosphate and base protecting groups, a monomethoxytrityl-NH$(CH_2)_3PO_4$ - oligomer is obtained. The monomethoxytrityl group can be removed with acetic acid to give the amine-containing oligonucleotide.

V.2.2. Chemical attachment of nucleotide derivatives to the termini of capture oligonucleotides

In another embodiment, nucleotide derivatives suitable for immobilization onto solid supports are incorporated into capture oligonucleotides during automated chemical oligonucleotide synthesis. For example, oligonucleotides may be derivatized at their 5'-terminus by coupling of a uridine moiety via a 5'-5' linkage using 2',3'-di-O-acetyluridine 5'-(2-cyanoethyl N,N-diisopropylphosphoramidite). (Kuijpers, W.H.A. et al., Bioconjugate Chem. 4, 94, 1993). After oxidation of the 2',3' cis-diol of the terminal uridine residue at the 5'-terminus by treatment with periodate, the derivatized oligonucleotide can be immobilized onto amine-containing solid supports via reductive amination. Other nucleotide derivatives such as N-6 or C-8 derivatives of dATP carrying protected amine or protected sulfhydryl groups are equally applicable for this invention.

V.2.3. Enzymatic attachment of nucleotide derivatives to the termini of capture oligonucleotides

In another embodiment, nucleotide derivatives containing reactive residues are incorporated into capture oligonucleotides by enzymatic means. Preferred examples of purine nucleotides include but are not limited to dATP derivatized with a reactive residue at its N-6 position or C-8 position via long linker arms. For example, 8-aminohexyl-dATP is a preferred derivative for coupling to the 3' terminal of DNA oligonucleotides by terminal transferase (Hermanson, G.T. (ed.) Bioconjugate techniques, Academic Press, San Diego, 1996). Preferred

examples of pyrimidine nucleotides include but are not limited to dUTP and dCTP modified with a reactive residue at their C-5 position via long linker arms.

V.2.4. Chemical modification of fold-back or looped preorganized capture oligonucleotides

Preorganized fold-back or looped capture oligonucleotides may be modified in the non-pairing nucleotide region with reactive groups by incorporation of nucleotide derivatives such as N-6 or C-8 derivatives of dATP carrying protected amine or protected sulfhydryl groups via long spacer arms. After deprotection, such derivatized preorganized fold-back or looped capture oligonucleotides can be immobilized onto amine- or sufhydryl-recative solid supports or further derivatized with heterobifunctional reagents. Alternatively, preorganized fold-back or looped capture oligonucleotides may be formed by non-nucleotide linker molecules providing a functional group for immobilization onto a solid support in addition to the other two functional groups required for the formation of a preorganized oligonucleotide structure by cross-linking of the termini. A convenient molecule from which to build trifunctional linker molecules is the amino acid L-lysine. Its three functional groups, α-carboxy, α-amino, and ε-amino, can be derivatized independently to contain three arms carrying different reactive groups.

V.2.5. Introduction of spacer molecules between solid support and capture oligonucleotides

In order to provide flexibility to oligonucleotides attached to solid supports, spacer molecules may be incorporated between the solid support and the oligonucleotide termini. Preferred spacer molecules are sufficiently long and flexible to allow efficient hybridization of the immobilized oligonucleotides with captured target nucleic acids. Preferred examples of spacer molecules are derivatives of 6-aminohexanoic acid (6-[6-((amino)hexanoyl) amino] hexanoate), providing a spacer arm with 14 atoms, oligomeric derivatives of 6-aminohexanoic acid (6-[6-((amino)hexanoyl)amino]-hexanoate), and oligoethylene glycol derivatives (Levenson, C., and Chang, C. Nonisotopically labelled probes and primers. In: PCR Protocols: A Guide to Methods and Applications (M.A. Innis et al., eds.) pp. 99-112, Academic Press, San Diego, 1990). Utilization of oligoethylene glycol as spacer molecules offers several advantages since oligoethylene glycol provides water solubility and a great degree of freedom for the linked oligonucleotides. Preferred are heterobifunctional derivatives of oligoethylene glycol including but not limited to those providing an amine-reactive residue such as a succinimidyl

ester on one end and a sulfhydryl-reactive residue such as a maleimide or vinylsulfone residue on the other end. Since vinylsulfone residues are hydrolytically stable, they represent especially useful reactive residues for a second coupling step. Useful are also derivatives of oligoethylene glycol containing a t-Boc-protected amine on one end and an unprotected amine or an amine-reactive residue on the other end. After reaction of the unprotected functional residue, the t-Boc protecting group can be easily removed by treatment with trifluoroacetic acid. Thus, a wide range of derivatives of oligoethylene glycol may be used as spacer molecules between the solid support and the oligonucleotide termini. Conjugation of capture oligonucleotides or derivatives thereof to spacer molecules may be accomplished by any of the well-known chemical coupling methods.

In one embodiment, primary amino groups are introduced at the 5'-termini of oligonucleotides by automated solid-phase synthesis using N-monomethoxytrityl-O-methoxydiisopropylaminophosphinyl-3-aminopropan(1)ol as mentioned above. Thereafter, the terminal amino group may be derivatized with an amine-reactive heterobifunctional reagent containing a long spacer arm such as succinimidyl 6-[3-(2-pyridyldithio) propionamido]hexanoate (LC-SPDP), succinimidyl 6-[6-{((iodoacetyl) amino) hexanoyl}amino]hexanoate (SIAXX), or oligoethylene glycol derivatives containing a succinimidyl ester on one end and a vinylsulfone residue on the other end. The sulfhydryl-reactive residue of such reagents may be used for coupling of the oligonucleotide-spacer conjugates to thiol-containing solid supports.

In another embodiment, nucleotide derivatives containing reactive residues for covalent attachment of spacer molecules are incorporated into oligonucleotides by enzymatic means. For example, 8-aminohexyl-dATP may be utilized for coupling to the 3' terminal of DNA oligonucleotides by terminal transferase (Hermanson, G.T. (ed.) Bioconjugate techniques, Academic Press, San Diego, 1996). The terminal amino group of the incorporated nucleotide may be further derivatized with heterobifunctional cross-linking reagents such as LC-SPDP, SIAXX, or oligoethylene glycol derivatives containing a succinimidyl ester on one end and a vinylsulfone residue on the other end. Using this approach, oligonucleotides can be derivatized at their 3'-terminus with a variety of spacer arms including 6-[6-((amino)hexanoyl)amino]-hexanoate and oligoethylene glycol derivatives of various length. The terminal sulfhydryl-reactive groups of such spacer molecules can be used for subsequent coupling of the oligonucleotide-spacer conjugates to

sulfhydryl-containing solid supports. Alternatively, the pyridyl disulfide residues of LC-SPDP-derivatized oligonucleotides can be reduced to generate free sulfhydryl groups for subsequent coupling of the oligonucleotide-spacer conjugates to sulfhydryl-reactive solid supports.

In another embodiment, oligonucleotides may be derivatized at their 5'-terminus by coupling of a uridine moiety via a 5'-5' linkage using 2',3'-di-O-acetyluridine-5'-(2-cyanoethyl-N,N-diisopropylphosphoramidite) (Kuijpers, W.H.A., et al, Bioconjugate Chem. 4, 94, 1993). After oxidation of the 2',3' cis-diol of the terminal uridine residue at the 5'-terminus by treatment with periodate, 6-[6-((amino)hexanoyl)amino]hexanoate or oligoethylene glycol derivatives containing an amine residue on one end and a carbyoxyl residue on the other end may be coupled to the aldehyde groups of the oligonucleotide via reductive amination. Thereafter, the terminal carboxyl residues of the spacer molecules may be activated for subsequent coupling of the oligonucleotide derivative to amine-containing solid supports.

In still another embodiment, preorganized fold-back or looped oligonucleotides are modified in the non-pairing nucleotide region with reactive groups by incorporation of nucleotide derivatives such as N-6 or C-8 derivatives of dATP carrying protected amine groups via long spacer arms (e.g., a protected derivative of 8-aminohexyl-dATP). After deprotection, such derivatized preorganized fold-back or looped oligonucleotides may be further derivatized with heterobifunctional cross-linking reagents such as LC-SPDP, SIAXX, or oligoethylene glycol derivatives containing a succinimidyl ester on one end and a vinylsulfone residue on the other end. Alternatively, preorganized fold-back or looped oligonucleotides are formed by non-nucleotide linker molecules such as L-lysine providing a functional group for covalent attachment of spacer molecules in addition to the other two functional groups required for formation of the preorganized oligonucleotide structure. Based on the successful use of hexaethylene glycol for the construction of looped oligonucleotides, preferred examples of trifunctional linker molecules are L-lysine residues in which the α-amino and ε-amino are derivatized with hydroxyl terminal spacer molecules and the α-carboxyl group with 6-[6-((amino)hexanoyl)-amino]hexanoate or oligoethylene glycol derivatives containing an amine residue on one end and a carboxyl residue on the other end.

V.3. AFFINTY COMPONENTS CAPABLE OF BINDING TO

CAPTURED NUCLEIC ACIDS

The liposomes to be used in the present invention contain encapsulated reporter molecules and are derivatized on the surface with affinity components capable of binding to captured target nucleic acids or derivatives thereof. Preferred affinity components include but are not limited to single-chain nucleic acids (ribo and deoxyribo nucleic acids), single-chain oligonucleotides (ribo and deoxyribo oligonucleotides), 'preorganized' oligonucleotide structures including peptide nucleic acid (PNA) analogues, intercalating agents, intercalating agents conjugated to oligonucleotides or nucleic acids, immunoglobulins or fragments of immunoglobulins with specificity for double- and/or triple-stranded nucleic acids), and nucleic acid binding proteins (e.g., the prokaryotic lac repressor or the eukaryotic cyclic AMP responsive element binding protein (CREB)).

V.3.1. Intercalating agents

In one preferred embodiment of liposome-coupled affinity components, DNA intercalating agents are used. Intercalating agents are molecules which are characterized by planar aromatic ring structures of appropriate size and geometry that can be inserted between baise pairs in double-stranded (ds) DNA. During intercalation neighboring base pairs in DNA are separated to allow for the insertion of the intercalating ring system, causing an elongation of the double helix by stretching. Examples include but are not limited to phenanthridines and acridines (e.g., ethidium bromide; propidium iodide; hexidium iodide; acridine orange; 9-amino-6-chloro-2-methoxyacridine (ACMA)), indoles and imidazoles (e.g., the bisbenzimide dyes Hoechst 33258 and Hoechst 33342; 4',6-diamidino-2-phenylindole (DAPI); 4',6-(diimidazolin-2-yl)-2-phenylindole (DIPI)), anthracyclines (e.g., doxorubicin; daunorubicin), cyanine dyes (e.g., benzoxazolium-4-pyridinium dyes, benzothiazolium-4-pyridinium dyes; benzoxazolium-4-quinolinium dyes; benzothiazolium-4-quinolinium dyes), actinomycin D, hydroxystilbamidine, psoralens (e.g., 4 ,5',8-trimethylpsoralen (trioxsalen); psoralen; angelicine (isopsoralen)), and derivatives thereof. The various classes of intercalating agents are characterized by different binding affinity constants as well as by different preferences for base-pairs and DNA conformations.

It is preferred that the DNA intercalating agents used as affinity components possess preference for double-stranded and/or triple-stranded DNA. Such DNA intercalating agents may be actinomycin D, anthracyclines, e.g. doxorubicin and daunorubicin, DAPI, and the bisbenzimide intercalators Hoechst 33258 and Hoechst 33342. DAPI associates in the minor groove of double-stranded DNA,

preferentially binding to AT clusters, although there is evidence that DAPI also intercalates in those DNA sequences containing as few as two AT base pairs. Binding of DAPI to double-stranded DNA occurs with an approximately 20-fold fluorescent enhancement, apparently due to the displacement of water molecules from both DAPI and the minor groove. Fluorescence enhancement does not occur with single-stranded DNA. The relatively non-toxic and water-soluble (2%) bisbenzimide intercalators Hoechst 33258 and Hoechst 33342 are also minor groove-binding DNA intercalators with a preference to contiguous AT base pairs.

Alternatively, homodimers and heterodimers of intercalating agents may be used as affinity components. It has been shown that appropriately designed dimers of intercalating agents have DNA binding affinities several orders of magnitude greater than those of their parent compounds. For example, the intrinsic DNA binding affinity of ethidium bromide and ethidium homodimer are 1.5×10^5 M^{-1} and 2×10^8 M^{-1}, respectively. Recently, several sets of dimers of intercalating agents have been developed (Haugland, R.P. Handbook of fluorescent probes and research chemicals, M-T.Z. Spence, ed., Molecular Probes, Inc., Eugene, OR, USA, 1996). For example, in the dimeric cyanine dye TOTO-1 (Molecular Probes, Inc., Eugene, OR, USA) the positively charged side chains of two monomeric cyanine dyes TO-PRO-1 (Molecular Probes, Inc., Eugene, OR, USA) are covalently linked. TOTO-1 exhibits a higher affinity for double-stranded DNA than even the ethidium homodimers. NMR studies of TOTO-1 interactions with a double-stranded 8-mer indicated that it is a bis-intercalator with interactions in the minor groove and that it distorts the helix by unwinding it where the dye is bound. In addition, TOTO-1 exhibits strong sequence selectivity for the site CTAG

In still another embodiment, intercalating agents covalently linked to oligonucleotides are used as affinity components. The oligonucleotides provide binding specificity by hybridizing to complementary single-stranded sequences of the target nucleic acids. In preferred assay procedures of this invention for detecting and quantifying target nucleic acids, capture oligonucleotides with a limited length are used. Thereby, only a part of the target nucleic acid is hybridized to the immobilized capture oligonucleotide, while the rest of the target nucleic acid remains in a single-stranded configuration. The intercalating agent should interact with the hydrid duplex structure formed by the nucleic acid target and the oligonucleotide-intercalating agent conjugate, provided the length of the linker is sufficient to allow for appropriate folding. This approach has been demonstrated with an acridine derivative (2-methoxy-6-chloro-9-aminoacridine)

covalently linked to the 3'-phosphate of a series of oligodeoxythymidylates via a polymethylene linker [$(Tp)_n(CH_2)_mACr$] (Héléne, C., et al., Biochem. Soc. Transact. 14, 201, 1986). NMR analyses revealed that the acridine ring was intercalated between AT base pairs. Additional stabilization was achieved when the 3'-phosphate group was twice substituted by the acridine derivative and a positively charged group to form a phosphotriester (Asseline, U., et al., J. Biol. Chem. 260, 8936, 1985). Thus, preferred examples of oligonucleotide-intercalator conjugates include conjugates in which intercalating agents with a preference for double-stranded and/or triple-stranded DNA are covalently linked to oligonucleotides via flexible oligomethylene linkers. In a more preferred embodiment, oligonucleotides containing a 3'-phosphate group and a uridine residue at the 5'-terminus via a 5'-5'-linkage (prepared by automated oligonucleotide synthesis as described by Kuijpers, see above) are used. The 2',3' cis-diol of the terminal uridine residue at the 5'-terminus is oxidized by treatment with periodate and the resulting aldehyde functions are used to attach long spacer molecules via reductive amination for covalent coupling of the oligonucleotide derivatives to the surface of affinity liposomes. The 3'-phosphate group is derivatized with an intercalating agent (with a preference for double-stranded and/or triple-stranded DNA) using a flexible oligomethylene linker.

V.3.1.1. Actinomycin D and derivatives

As mentioned above, derivatives of actinomycin D may be used as intercalating agent. Actinomycin D has a phenoxazin-2-amino-3-one chromophore and two cyclic pentapeptide lactones attached to the 1,9-positions of the chromophore. It binds to dG-dC base pairs in double-standed DNA by intercalation of its chromophore and by hydrogen bonding and hydrophobic interactions of its peptide lactones. The unsubstituted 3-oxo, and 4- and 6-methyl groups of the chromophore as well as the integrity of the cyclic pentapeptide lactones are necessary for the biochemical properties of actinomycin D. However, systematic studies of structural modifications have demonstrated that the molecule of actinomycin D can accomodate a number of well-defined modifications at the C-7- and N^2-sites while retaining its biochemical properties (Sengupta, S.K., et al., J. Med. Chem. 24, 1052, 1981). Even after substitution at the C-7-position with bulky groups via rotationally flexible linkers, the DNA binding properties of actinomycin D are retained. For example, actinomycin D and the actinomycin D analogue 7-[(3,4-dichlorobenzyl)amino]-actinomycin D intercalate into calf thymus DNA with apparent binding constants of $2.3 \times 10^7\ M^{-1}$ and $2.6 \times 10^7\ M^{-1}$, respectively. Apparently, the flexible NH-CH_2 linkage of the actinomycin D

analogue allows for the formation and aids the stabilization of the intercalated complex with DNA. Preferred examples of actinomycin D derivatives for the preparation of affinity liposomes include but are not limited to actinomycin D analogues containing a reactive residue attached to the N^2- or C-7-position via rotationally flexible linker molecules.

V.3.1.2. Anthracyclines and derivatives

Further, derivatives of the anthracyclines doxorubicin and daunorubicin may be used as intercalating agents for the preparation of affinity liposomes. Doxorubicin is a glycoside antibiotic that differs from daunorubicin by a single hydroxyl group on C-14. Both molecules contain an aminosugar, daunosamine, linked through a glycosidic bond to the naphthacenequinone nucleus. They intercalate into the DNA double helix in such a fashion that the aglycone moiety is between the adjacent base pairs and parallel to them. The daunosamine moiety lies in the major groove of the double helix and the protonated amine of the aminosugar binds electrostatically to the negatively charged phosphate groups of the DNA. Both antracyclines intercalate into the DNA double helix with a binding affinity constant of approximately 10^6 M^{-1}, which is the same order of magnitude as those found for actinomycin and the acridines.

It is known that doxorubicin as well as daunorubicin derivatives containing a daunosamine moiety with a modified or substituted amino group are still able to form complexes with DNA. For example, the N-acetyl derivative of daunorubicin binds to DNA, athough less strongly than non-derivatized daunorubicin. The 3'-deamino-3'-(3-cyanomorpholinyl)-derivative of doxorubicin represents another example. In dilution experiments, the complex of this compound with DNA exhibits a typical irreversible binding (Menozzi, M. et al., Stud. Biophys. 104, 113, 1984). Furthermore, the antracyclines cinerubin A and B containing pyrromycinone as aglycone and a trisaccharide instead of a monosaccharide are also capable of intercalating into DNA. Despite the presence of the bulky trisaccharide moiety, the stiffening and elongating effects of cinerubins on DNA resemble the changes produced by acridine dyes. Thus, examples of doxorubicin and daunorubicin derivatives for the preparation of affinity liposomes are derivatives containing a flexible spacer attached to the daunosamine moiety. In a preferred embodiment, the amine group of the daunosamine moiety is derivatized with 2-iminothiolane (Jue, R. et al., Biochemistry 17, 5399, 1978). The cyclic imidoester reacts with amines to form a stable, positively charged linkage and leaves the sulfhydryl group available for further coupling. Using this heterobifunctional reagent, the

positive charge of the original amine is preserved and can bind electrostatically to the negatively charged phosphate groups of the DNA.

V.3.2. Oligonucleotides and oligonucleotide derivatives

In another preferred embodiment, single chain oligonucleotides (ribo and deoxyribo oligonucleotides) and oligonucleotide derivatives are employed as affinity components. The oligonucleotides are designed to form specific helical complexes with those regions of captured target nucleic acids that are still in a single-stranded configuration. In a more preferred embodiment, 'preorganized' oligonucleotide structures are used as affinity components. The rational for using such structures has been illustrated in detail above (in connection with the description of capture oligonucleotides).

In order to provide flexibility to oligonucleotides attached to the surface of affinity liposomes, spacer molecules will usually be incorporated between the liposomal surface and the oligonucleotide termini. Preferred spacer molecules are sufficiently long and flexible to allow efficient hybridization of the tethered oligonucleotides with captured target nucleic acids or amplicons thereof. Preferred examples of spacer molecules include those which have been described above in connection with the introduction of spacer molecules between solid support and capture oligonucleotides.

Derivatization of the oligonucleotides or derivatives thereof with spacer molecules may be accomplished by any of the well-known chemical coupling methods. Preferred methods may be e.g. those which allow selective derivatization of the termini to guarantee efficient hybridization with target nucleic acids.

In one preferred embodiment of oligonucleotide derivatization with spacer molecules, oligonucleotides containing a 5'-phosphate group are derivatized in a carbodiimide-mediated reaction with derivatives of oligoethylene glycol containing a t-Boc-protected amine on one end and an unprotected amine on the other end. First, the 5'-phosphate groups are reacted with carbodiimide in the presence of imidazole to form active phosphorimidazolide intermediates. (Hermanson, G.T. Bioconjugate techniques, Academic Press, San Diego, 1996). These derivatives are highly reactive towards amine nucleophiles such as amine derivatized oligoethylene glycol spacer molecules. Thereafter, the terminal t-Boc protecting group is removed by treatment with trifluoroacetic acid for subsequent coupling of

the amine terminal oligonucleotide-spacer conjugates to amine-reactive lipid derivatives in the liposomal bilayer.

In another preferred embodiment, primary amino groups are introduced at the 5'-termini of oligonucleotides by automated solid-phase synthesis using N-monomethoxytrityl-O-methoxydiisopropylaminophosphinyl 3-aminopropan (1) ol (Connolly, B.A. Nucleic Acids Res. 15, 3131, 1987). Thereafter, the terminal amino group may be derivatized with an amine-reactive heterobifunctional reagent containing a long spacer arm (LC-SPDP), (SIAXX), or oligoethylene glycol derivatives containing a succinimidyl ester on one end and a vinylsulfone residue on the other end. The sulfhydryl-reactive residue of such reagents may be used for coupling of the oligonucleotide-spacer conjugates to thiol-containing lipid molecules (e.g., 2-iminothiolane-derivatized phosphatidyl ethanolamine) in the liposomal bilayer. .

In another preferred embodiment, nucleotide derivatives containing reactive residues for covalent attachment of spacer molecules are incorporated into oligonucleotides by enzymatic means. Preferred examples of modified nucleotides include those derivatives in which the reactive residue is incorporated in a way that does not affect enzyme recognition and activity. Preferred examples of the purine nucleotides have been detailed above under "Enzymatic attachment of nucleotide derivatives to the termini of capture oligonucleotides". Preferred examples of the pyrimidine nucleotides include but are not limited to dUTP and dCTP modified with a reactive residue at their C-5 position via long linker arms. In a more preferred embodiment, 8-aminohexyl-dATP is utilized for coupling to the 3' terminal of DNA oligonucleotides by terminal transferase (Hermanson, G.T. (ed.) Bioconjugate techniques, Academic Press, San Diego, 1996). The terminal amino group may further be derivatized with LC-SPDP. Using this approach, the oligonucleotides are derivatized at their 3'-terminus with spacer arms which are similar in length as 6-[6-((amino)hexanoyl)amino]-hexanoate. The terminal pyridyl disulfide groups can be used for subsequent coupling of the oligonucleotide derivative to sulfhydryl-containing lipid molecules in the liposomal bilayer. Alternatively, the pyridyl disulfide residues can be reduced to generate free sulfhydryl groups for subsequent coupling of the oligonucleotide derivative to sulfhydryl-reactive lipid molecules in the liposomal bilayer (e.g., pyridyl disulfide-, maleimide- or iodoacetyl-containing derivatives of phosphatidyl ethanolamine).

In still another preferred embodiment, oligonucleotides are derivatized at their 5'-terminus during automated chemical oligonucleotide synthesis by coupling of a uridine moiety via a 5'-5' linkage using 2',3'-di-O-acetyluridine 5'-(2-cyanoethyl N,N-diisopropylphosphoramidite) as detailed above for chemical attachment of nucleotide derivatives to the termini of capture oligonucleotides. After the periodate treatment of the terminal uridine residue, 6-[6-((amino)hexanoyl) amino]hexanoate is coupled to the aldehyde groups of the oligonucleotide via reductive amination. Thereafter, the terminal carboxyl residues of the spacer molecules are activated with N-hydroxysuccinimide in a carbodimide-mediated reaction for subsequent coupling of the oligonucleotide derivative to amine-containing lipid molecules (e.g., phosphatidyl ethanolamine) in the liposomal bilayer.

In still another preferred embodiment, preorganized fold-back or looped oligonucleotides are modified in the non-pairing nucleotide region with reactive groups during automated chemical oligonucleotide synthesis by incorporation of nucleotide derivatives such as N-6 or C-8 derivatives of dATP carrying protected amine groups via long spacer arms (e.g., a protected derivative of 8-aminohexyl-dATP). After deprotection, such derivatized preorganized fold-back or looped oligonucleotides are further derivatized with the heterobifunctional cross-linking reagent LC-SPDP. The terminal pyridyl disulfide groups can be used for subsequent coupling of the oligonucleotide derivative to sulfhydryl-containing lipid molecules in the liposomal bilayer. The pyridyl disulfide residues can also be reduced to generate free sulfhydryl groups for subsequent coupling of the oligonucleotide derivative to sulfhydryl-reactive lipid molecules in the liposomal bilayer (e.g., pyridyl disulfide-, maleimide- or iodoacetyl-containing derivatives of phosphatidyl ethanolamine). Alternatively, preorganized fold-back or looped oligonucleotides are formed by non-nucleotide linker molecules providing a functional group for covalent attachment of spacer molecules in addition to the other two functional groups required for formation of the preorganized oligonucleotide structure. A preferred molecule for the construction of such trifunctional linker molecules is the amino acid L-lysine. Its three functional groups, α–carboxy, α-amino, and ε-amino, can be derivatized independently to contain three spacer arms wth different terminal reactive groups. Based on the successful use of hexaethylene glycol for the construction of looped oligonucleotides, preferred example of trifunctional linker molecules include L-lysine residues inwhich the α-amino and ε-amino are derivatized with hydroxyl

terminal spacer molecules and the α-carboxyl group with 6-[6-((amino)hexanoyl)-amino]hexanoate in a carbodiimide-mediated reaction.

V.3.3. Antibodies with specificity for double-stranded and/or triple-standed *nucleic acids*

In another preferred embodiment, antibodies with specificity for double and/or triple helical structures of DNA are employed as liposome-attached affinity components. The term "antibody" refers to immunoglobulins of any isotype or subclass as well as any fragment (e.g., Fab' of Fv fragments) of the aforementioned. Antibodies of any source are applicable including polyclonal materials obtained from any animal species, monoclonal antibodies from any hybridoma source, and all immunoglobulins (or fragments) generated with the aid of viral, prokaryotic or eukaryotic expression systems. Biologic recognition molecules with specificity for double and/or triple helical structures of DNA other than antibodies are equally applicable for use with the current invention.

V.4. REPORTER MOLECULES

As mentioned above, suitable reporter molecules comprise those which may undergo oxidation or reduction and preferably are capable to act as redox mediators.

V.4.1. Reporter molecules capable of being oxidized or reduced

Reporter molecules which may be electrochemically oxidized or reduced comprise hydrochinones, napthtols or organometals, e.g. organometallic complexes of osmium, ruthenium, cobalt and others.

V.4.2. Redox mediators

In a preferred embodiment, redox mediators are used as reporter molecules which are susceptible to redox recycling in closely spaced arrays of thin film nobel metal electrodes. Redox mediators are more sensitive than reporter molecules which may only be oxidized or reduced Preferred redox mediators of this invention are characterized by several important properties. To allow for efficient encapsulation in affinity liposomes, preferred redox mediators possess high solubility in aqueous media. The redox potentials should exhibit reversible peaks in cyclic voltammograms and in aqueous solutions the potentials should be within the limits of - 600 mV (generation of oxygen) and + 800 mV (generation of hydrogen). Preferred are potentials close to 0 mV since they guarantee minimal background

current as well as minmal instrumental background noise of the electrode, thereby providing optimal signal-to-noise ratios. Potentials close to 0 mV provide an additional advantage in that they minimize or avoid interference by electroactive compounds present in samples to be analyzed such as ascorbic acid in blood or catecholamines in urine.

One preferred class of redox mediators contains one or more aromatic ring structures and various organic substituents and side chains that allow switching from a regular aromatic ring structure to a quinone structure and vice versa. The benzoquinone/hydoquinone couple and the p-aminophenol/quinoneimine couple are well known redox couples of this class. Other preferred examples of this class of redox mediators include but are not limited to catechol and catechol derivatives (e.g., adrenalin, dihydroxyphenylalanine, epinine, adrenalone, norhomoepinephrine, and protocatechnic acid), dopamine, methoxytyraminin, aromatic compounds with more than one aromatic ring structure such as anthracene derivatives, as well as heterocyclic aromatic compounds such as serotonin and hydroxyindolacetic acid. Aromatic redox mediators may be further derivatized with appropriate substituents to generate optimal redox potentials.

Another preferred class of redox mediators are organic and anorganic metal complexes that can be reversibly oxidized and reduced. In a more preferred embodiment, the metal complexes contain osmium, ruthenium, iron, copper, or chromium. Some of these metal complexes such as the ferricinium ion/ferrocene couple and bipyridyl derivatives of the Os(II)/Os(III) couple have been employed in numerous amperometric and voltammetric assay procedures. Metal complexes provide several important advantages as redox mediators. Most important, metals can be complexed by various aromatic and heterocyclic aromatic compounds, the side chains of which may be used to adjust the redox potential according to the specific assay requirements. For example, the redox potential of ferrocene has been changed to both more anodic and more cathodic redox species by derivatization with residues such as carboxyl groups, halogens, aminoethyl groups, or pyridine derivatives. Preferred are electron positive side chains since they tend to change the potential of redox mediators towards O mV. Metal complexes provide a second important advantage in that they offer the possibility to introduce water-solubility by derivatization of the complexes with polar residues or groups that can be protonated. For example, covalent derivatization of the phenyl residues of Os-bipyridyl-complexes with carboxyl groups improve the water-solubility and, thereby, the access of theses complexes to the electrodes by

a factor of 10 as compared to Os-bipyridyl-complexes formed with non-derivatized, hydrophobic phenyl residues. As a result, carboxyl-derivatized Os-bipyridyl-complexes provide both, high water-solubility and highly efficient redox reycling.

V.5. AFFINITY LIPOSOMES

In the present invention, captured analytes are detected by affinity liposomes containing encapsulated reporter molecules, preferably redox mediators. Affinity components attached to the liposomal surface are utilized to mediate analyte-specific binding of affinity liposomes. The release of redox mediators encapsulated in specifically bound affinity liposomes is dependent of the kind of liposomes selected; often it may be effected by an increase of the ambient temperature or the addition of liposome lysing solvents (e.g., detergents).

VII.5.1. Choice of liposomes

Liposomes are artificial structures primarily composed of phospholipid bilayers exhibiting amphiphilic properties. Other molecules, such as cholesterol, fatty acids, or lipid derivatives also may be included in the bilayer construction. The morphology of liposomes can be classified according to compartmentalization of aqueous regions between bilayer shells. If the aqueous regions are segregated by only one bilayer each, the liposomes are called unilamellar vesicles (ULV). If there is more than one bilayer surrounding each aqueous compartment, the liposomes are termed multilamellar vesicles (MLV). ULV are further classified according to their relative size. Usually, the diameter of small unilamellar vesicles (SUV) is less than 100 nm with a minimum of about 25 nm, whereas the diameter of large unilamellar vesicles (LUV) is more than 100 nm with a maximum of about 2500 nm. MLVs typically form large complex honeycomb structures. As a consequence of the almost infinite number of ways each bilayer can be associated and interconnected with other bilayers, MLVs are difficult to categorize or exactly to reproduce. MLVs are the simplest to prepare, the most stable, and the easiest to scale up to large production levels. Although the many kinds of liposomes may be used in the present invention, the most useful form thereof consists of small, spherical ULVs containing hydrophilic electrochemically active reporter molecules that are protected from the outer environment by the lipid shell.

The outside surface of the liposomes of the present invention is derivatized to contain covalently attached molecules designed to target the liposomes for

specific interactions with nucleic acids or derivatives thereof. Such liposomes are termed affinity liposomes in this invention.

V.5.2. Lipid components of affinity liposomes

Phospholipids are the most important constituents of affinity liposomes. Two main forms of lipid derivatives exist biologically, molecules containing a glycerol backbone and those containing a sphingosine backbone. Naturally occurring phospholipids can be isolated from a variety of sources including egg yolk. The composition of egg phospholipids, however, can vary considerably depending on age of the eggs, the diet of the chickens, and the method of processing. Furthermore, egg lecithin for example is not a single compound, but consists of a mixture of phosphatidyl cholines containing about 31% saturated fatty acid having a chain length of 16 carbons, 16% saturated fatty acid with 18 carbons, about 48% also with 18 carbons but having at least 1-2 points of unsaturation, and the rest a variety of other fatty acid constituents. Preferred for this invention are synthetic phospholipids of known chemical purity. Three major fatty acid derivatives of synthetic phospholipids are used primarily in affinity liposome preparation: (1) myristic acid (n-tetradecanoic acid; containing 14 carbons), (2) palmitic acid (n-hexadecanoic acid; containing 16 carbons), and (3) stearic acid (n-octadecanoic acid, containing 18 carbons).

Another significant component of affinity liposome preparations may be cholesterol. The presence of cholesterol in affinity liposome membranes has the effect of decreasing or even abolishing (at high cholesterol concentrations) the phase transition from gel state to the fluid or crystal state that occurs with increasing temperature. As a result, cholesterol modulates the permeability and fluidity of the associated membrane, increasing both parameters at temperatures below the phase transition point and decreasing both above the phase transition temperature.

V.5.3. Lipid composition of affinity liposomes

One group of lipid compositions for the preparation of stable affinity liposomes of the present invention contains phosphatidyl ethanolamine (PE) derivatives. Examples include compositions with molar ratios of phosphatidyl choline (PC): cholesterol: negatively charged phospholipid (e.g., phosphatidyl glycerol, PG): derivatized PE of 8: 10: 1: 1. Another preferred composition using a maleimide derivative of PE without PG is PC: cholesterol: maleimide-PE of 85: 50: 15 (Friede, M. et al., F. Anal. Biochem. 211, 117, 1993). In a more preferred

embodiment of this invention, the PE derivatives do not exceed a ratio of 1-10 mol PE per 100 mol of total lipid to maintain membrane stability.

Another group of liposomes may be used in the present invention which allow the release of encapsulated reporter molecules, e.g. redox mediators, by a moderate increase of the ambient temperature. Liposomes are known to release encapsulated water-soluble contents more quickly near their liquid crystalline phase-transition temperature (T_m) than at other temperatures (Blok, M.C. et al., J. Biochim. Biophys. Acta 433, 1, 1976). Such a temperature-sensitive release can be engineered by the selection of pure lipids that undergo sharp transition temperatures or by using mutually miscible mixtures of pure lipids to adjust the transition temperature to the desired point. Examples of such lipids are dipalmitoyl phosphatidylcholine (DPPC) (T_m = 41 °C), dipalmitoyl phosphatidyglycerol (DPPG) (T_m = 41 °C), and distearoyl phosphatidylcholine (DSPC) (T_m = 54 °C). The choice of lipids and the relative proportion of each depend upon the desired T_m and the size of the liposome. SUV have apparent transition temperatures several degrees below those predicted from the T_m of the component lipids in LUV or MLV. This effect is probably a result of stress in the highly curved bilayer structure in SUV. Typical lipid compositions of temperature-sensitive SUV, LUV, and MLV liposomes are described by various authors (for example, see, Magin, R.L., and Weinstein, J.N. In: Liposome Technology. (G. Gregoriadis, ed.) vol. III., pp. 137-155, CRC Press, Boca Raton, Fl., 1984).

V.5.4. Methods for the preparation of affinity liposomes

Several methods are available to prepare affinity liposomes which are useful for this invention. One group of them comprises the following steps: (i) dissolving the lipid mixture in organic solvent, (ii) dispersion in an aqueous phase containing redox mediators, and (iii) fractionation to isolate the correct liposomal population. During all handling procedures the solutions are preferably protected from excessive exposure to light. Organic solvents are preferably maintained under a nitrogen or argon atmosphere to prevent introduction of oxygen. Water and buffers are preferably degassed using a vacuum and bubbled with inert gas before lipid components are introduced.

In the first step of these methods, the lipid components are dissolved in organic solvent (e.g., chloroform: methanol, by volume 2:1). This mixture will include any phospholipid derivatized with reactive groups or affinity components soluble in

organic solvents as well as other lipids used to form the liposomal structure. Once the desired mixture of lipid components is dissolved and homogenized in organic solvent, one of several techniques may be used to disperse the liposomes in aqueous solution containing the redox mediators. Preferred methods include (i) mechanical dispersion, (ii) detergent-assisted solubilization, and (iii) solvent-mediated dispersion.

Using mechanical dispersion methods for the preparation of affinity liposomes, an aqueous solution containing the redox mediators is added to the dried, homogenous lipid mixture and manipulated to effect dispersion. In a preferred embodiment, mechanical dispersion methods include but are not limited to simple shaking, non-shaken aqueous contact, high-pressure emulsification, sonication, extrusion through small-pore membranes, and various freeze-thaw techniques. Most of these methods result in a population of vesicles ranging from SUVs of only 25 nm diameter to very large MLVs.

Using detergent-assisted dispersion methods for the preparation of affinity liposomes, the amphipathic nature of detergent molecules is utilized to bring more effectively the lipid components into the aqueous phase for dispersion. The detergent molecules bind and mask the hydrophobic tails of lipids from the surrounding water molecules of the aqueous phase containing the redox mediators. Detergent treatment may be performed using a dried lipid mixture or small vesicles. In a preferred embodiment, non-ionic detergents such as the Triton X family, alkyl glycosides, or bile salts such as sodium deoxycholate are employed for this procedure. On removal of the detergent from the solution, the lipid micelles aggregate to form larger liposome structures. Liposomes of up to 100 nm containing a single bilayer may be created using detergent-asssisted methods.

Using solvent-mediated dispersion techniques for the preparation of affinity liposomes, the lipid mixture is first dissolved in a organic solvent to create a homogeneous solution and thereafter introduced into a aqueous phase containing the redox mediators. The solvent may or may not be soluble in the aqueous phase to effect this process. One preferred example of a solvent-mediated dispersion method is described by Batzri, S., and Korn, E.D. (Biochim. Biophys. Acta 298, 1015, 1973). Phospholipids and other lipids to be part of the liposomal membrane are first dissolved in ethanol. This ethanolic solution is then rapidly injected into a redox mediator-containing aqueous solution of 0.16 M KCl using a

Hamilton syringe, resulting in a maximum concentration of no more than 7.5% ethanol. Using this method, single bilayer liposomes of about 25 nm diameter can be formed. Other preferred solvent-mediated dispersion methods utilize solvents that are insoluble in the aqueous phase The production of liposomes by this procedure involves the formation of a 'water-in-oil' emulsion. To create a proper reverse-phase emulsion, a small quantity of aqueous phase containing the redox mediators is introduced into a large quantity of organic phase containing the dissolved liposomes. The result is a milky dispersion. The emulsification process involves the use of mechanical means (shaking, stirring, or sonication) to effect the formation of small droplets of aqueous solution uniformly dispersed in the lipid-organic phase. Excess of organic solvent is then removed by rotary evaporation (reverse-phase-evaporation method) until the mixture becomes a viscous gel. To facilitate liquification of the gel, a small volume of aqueous phase is added. Finally, residual organic solvent and untrapped redox mediators are removed by dialysis.

V.5.5. Encapsulation and release of reporter molecules from affinity liposomes

The encapsulation efficiency of reporter molecules, preferably water-soluble redox-mediators, within affinity-liposomes depends on the liposome type. A comparative analysis of the encapsulation effiency in SUVs, LUVs, and MLVs of the water-soluble marker compounds 5(6)-carboxyfluorescein (CF) and tritiated cytosine-1-β-D-arabinofuranoside ([^{3}H]-Ara-C) has been performed by Magin, R.L., and Weinstein, J.N. (In: Liposome Technology (G. Gregoriadis, ed.) vol. III., pp. 137-155, CRC Press, Boca Raton, Fl., 1984). Typical values of this study are given in Table I. For a fixed quantity of lipid, LUVs have the highest encapsulation efficiency.

In order to prepare liposomes which contain the said reporter molecules, a suitable mixture of lipids is provided to obtain a stable liposomal layer which is preferably solved in an organic solvent. One method to prepare the said liposomes is to dry the lipid mixture, e.g. under reduced pressure at or close to ist transition temperature (the highest transition temperature of any one lipid in the mixture is taken into consideration) and to hydrate the lipid mixture with the reporter molecules dissolved in a suitable aqueous buffer. Vortexing or sonification assists the forming of liposomes. Another method to prepare the said liposomes is to provide the lipid mixture in a suitable organic solvent, to add the reporter molecules in an aqueous buffer, to sonicate the mixture until it appears

homogeneous and to evaporate the organic phase, e.g. under reduced pressure (reverse-phase evaporation technique). The size of the liposomes thus obtained may be modified, e.g. SUV`s filled with enzyme activator may be converted into LUV`s. The liposomes may be separated from excess enzyme activator in their environment by a variety of methods, for example using gel chromatography or washing steps. Alternatively or in addition, they may be centrifuged or dialyzed against buffer. Further, the liposomes may be filtered, e.g. under pressure or with suction through a 0.3 to 1.0 µm pores containing filter. Via filtering, not only a separation from excess enzyme activator is possible, but also controlling their size and/or size distribution.

The release of encapsulated reporter molecules may be mediated by the addition of detergent (i.e., Triton X-100 or sodium deoxycholate) or organic solvent. Further, the release of encapsulated reporter molecules may be mediated by an increase of the ambient temperature (phase-transition release). A comparative analysis of phase-transition-mediated release of water-soluble marker compounds from different types of temperature-sensitive liposomes has been performed by Magin, R.L., and Weinstein, J.N. (The design and characterization of temperature-sensitive liposomes. In: Liposome Technology (G. Gregoriadis, ed.), vol. III., pp. 137-155, CRC Press, Boca Raton, Fl., 1984). Typical values obtained with the marker compounds 5(6)-carboxyfluorescein (CF) and tritiated cytosine-1-β-D-arabinofuranoside ([^{3}H]-Ara-C) are summarized in Table II below. Preferred heating rates at passage through T_m are 10 to 15 °C/min. Based on these data, detergent-mediated release of encapsulated redox mediators may be the preferred mechanism when ultimate detection sensitivity is required. The addition of detergent, however, requires to provide an additional container, whereas heating to approximately 45 °C is relatively easy to accomplish. Therefore, phase-transition-mediated release may be the preferred choice of release mechanism for less sensitive but for more cost-efficient assay procedures.

Table I. Encapsulation and release characteristics of different types of temperature-sensitive liposomes

	LIPOSOME TYPE		
	SUV (DPPC:DSPC) (7:3)	LUV (DPPC:DPPG) (4:1)	MLV (DPPC:DPPG) (4:1)
Diameter (nm)	20 - 50	70 - 800	125 – 2000
Captured volume (l/mg lipid)	0,1 - 0,6	7 – 12	5 – 7
Encapsulation efficiency (%)	0,2 – 2,0	20 – 40	15 - 20
Release (%) (1 min at 42°C)	5 - 10	30 - 60	30 – 60

DPPC, dipalmitoyl phosphatidylcholine (T_m = 41 °C); DPPG, dipalmitoyl phosphatidylglycerol (T_m = 41 °C); DSPC, distearoyl phosphatidylcholine (T_m = 54 °C)

V.6. METHODS FOR COVALENT COUPLING OF AFFINITY COMPONENTS TO LIPOSOMES

Different methods are available for covalent coupling of affinity components to liposomes. First, a purified lipid component (e.g., phosphatidyl ethanolamine or cholesterol) may be derivatized with an affinity component prior to incorporation into the lipid bilayer construction. Second, a purified lipid component may be activated prior to incorporation into the lipid bilayer construction and further derivatized with an affinity component after formation of the intact liposome. Third, the activation process and subsequent coupling step of an affinity component may be performed after formation of the intact liposome.

Numerous methods are available for derivatization of lipid functional groups. However, three main strategies are preferably used to couple affinity components to purified lipid components or lipid components incorporated into the lipid bilayer of liposomes: (i) heterobifunctional cross-linker-mediated conjugation reactions, (ii) carbodiimide-mediated reactions for coupling of amine residues to carboxyl groups or amine residues to phosphate groups, and (iii) reductive amination reactions for coupling of amine residues to aldehydes.

V.6.1. Activation of lipid components with heterobifunctional reagents

The most common type of heterobifunctional reagents for the activation of lipid components includes amine- and sulfhydryl-reactive cross-linking reagents containing an N-hydroxy-succinimide (NHS) ester on one end and a maleimide, iodoacetyl, or pyridyl disulfide group on the other end. Examples of reagents used for activation of lipid components include but are not limited to succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC), m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), N-succinimidyl-4-(p-maleimido-phenyl)butyrate (SMPB), N-succinimidyl-(4-iodoacetyl)-aminobenzoate (SIAB), N-succinimidyl-6-[(iodoacetyl)amino]hexanoate (SIAX), and N-succinimidyl-3-(2-pyridyl-dithio)propionate (SPDP). It is preferred to use cross-linking reagents which contain long spacer molecules between the two functionalities. The length of the spacer is important in providing sufficient distance from the liposomal surface to ensure binding of the liposome-attached affinity component with another affinity partner. Preferred examples of heterobifunctional cross-linking reagents with long spacer molecules include but are not limited to the long-chain versions of regular cross-linking reagents such as (LC-SPDP) and (SIAXX) (formulas see V.2.5).

In one preferred embodiment, phosphatidyl ethanolamine (PE) used as a component of the liposomes is derivatized with heterobifunctional cross-linking reagents, preferably with those which derivatize the amino group of PE with a pyridyl disulfide group (e.g., by reaction with LC-SPDP), a maleimide residue (e.g., by reaction with SMPB, an iodoacetyl group (e.g., by reaction with SIAXX), a thioester moiety (e.g., by reaction with SATP, succinimidyl acetyl-thiopropionate), or an aldehyde function (e.g., by reaction with SFPA, succinimidyl-p-formylphenoxyacetate). Purified PE phospholipid may be modified in organic solvent prior to incorporation into liposomes, or intact liposomes containing PE may be activated while suspended in an aqueous solution. It is more preferred that the PE derivative is prepared before the liposome is constructed. In this way, a stable stock preparation of modified PE can be made and used in a number of different liposome recipes. In a preferred embodiment, the PE employed is of a synthetic variety having fatty acid constitutents of dimyristoyl (DMPE), dipalmitoyl (DPPE), or distearoyl (DSPE) forms. For activation of pure PE, heterobifunctional reagents are preferred which are not modified with a sulfo residue at the N-hydroxysuccinimide ester moiety, since activation of PE is performed under non-aqueous conditions. If reporter molecules, e.g. redox mediators to be entrapped within the liposome are reactive with the PE derivatives, PE molecules are

activated after formation of the liposomal structures to ensure derivatization of only the outer half of the lipid bilayer. In this case, heterobifunctional reagents are preferred which are modified with a sulfo residue at the N-hydroxysuccinimide ester moiety, since sulfo-derivatized cross-linking reagents cannot penetrate lipid bilayers.

V.6.2. Coupling of affinity components to activated lipid components

Examples of useful derivatives of affinity components for covalent attachment to activated lipid components such as PE derivatives include but are not limited to daunorubicin derivatized at its daunosamine moiety with (LC-SPDP), (SIAXX), or 2-iminothiolane (2-IT), N^2-(3'aminopropyl) actinomycin D derivatized at its terminal amino group with LC-SPDP, SIAXX, or 2-IT, and 5'-amine terminal oligonucleotides derivatized with LC-SPDP or SIAXX.

The methods by which derivatives of affinity components may be covalently coupled to cross-linker-derivatized lipid components are numerous and well known in the art. For example, affinity components containing nucleophilic moieties such as a primary amine or a thiol may be reacted with lipid components that have been derivatized with an electrophilic moiety. Examples of electrophilic moieties include but are not limited to alkyl halides, alkyl sulfonates, active esters such as N-hydroxysuccimide esters, aldehydes, ketones, isothiocyano, maleimido, pyridyl disulfide, and carboxylic acid chloride residues. Vice versa, lipid components or lipid derivatives containing a nucleophilic moiety can be reacted with an electrophilic moiety on the affinity component. Thus, any of a wide range of functional groups may be utilized for conjugation provided these groups are complementary.

V.6.3. Coupling of affinity components to intact liposomes

Derivatization of lipid components after formation of intact liposomes is the preferred method of this invention, if proteinaceous affinity components such as antibodies with specificity for double- and/or triple-stranded nucleic acids are covalently attached to the liposomal surface or reporter molecules to be entrapped within the liposome are reactive with activated lipid components. In the latter case, lipid components such as PE molecules are activated after formation of the liposomal structures to ensure derivatization of only the outer half of the lipid bilayer. The methods by which affinity components may be covalently coupled to intact liposomes are similar to those employed for derivatization of lipid components and well known in the art. The sulfo-N-succinimidyl ester (sulfo-NHS)

variety of cross-linking reagents is preferred for activation of intact liposomes in aqueous suspension since they are incapable of penetrating membranes. Thus, only the outer surface of the liposomes will be modified.

Covalent attachment of proteinaceous affinity components (termed affinity proteins) such as antibodies may be performed with homobifunctional or heterobifunctional cross-linking reagents, with carbodiimides, by reductive amination, or by N-hydroxysuccinimide (NHS) ester activation of carboxylates. In one embodiment of the invention, affinity proteins are coupled via their primary amine groups to N-hydroxysuccinimide ester-derivatized palmitic acid followed by incorporation of the protein-palmitate conjugates into the bilayer construction of intact liposomes by detergent dialysis as described by Huang, A., Huang, L., and Kennel, S.J. (J. Biol. Chem. 255, 8015, 1980). In another embodiment, affinity proteins are coupled via their primary amine groups to DMS (dimethyl suberimidate)-derivatized PE molecules incorporated into the bilayer construction. DMS is a homobifunctional cross-linking agent containing amine-reactive imidoesters on both ends. The resulting amidine linkages are positively charged at neutral pH, thus maintaining the positive charge contribution of the original amine. In another preferred embodiment, affinity proteins are first derivatized with amine-reactive heterobifunctional cross-linking reagents to introduce pyridyl disulfide groups (e.g., by reaction with with LC-SPDP), maleimide residues (e.g., by reaction with succinimidyl 4-(p-maleimidophenyl)butyrate (SMPB)), or iodoacetyl groups (e.g., by reaction with SIAXX). Such derivatized affinity proteins are coupled to sulfhydryl-derivatized PE molecules incorporated into the bilayer construction. Vice versa, affinity proteins containing a free sulfhydryl group are coupled to incorporated PE molecules derivatized with sulfhydryl-reactive residues such as pyridyl disulfide, maleimide, or iodoacetyl groups. Affinity proteins without a free sulfhydryl group are first derivatized with amine-reactive heterobifunctional cross-linking reagents to introduce free or protected sulfhydryl residues such as thioester moieties (e.g., by reaction with SATP). The thioester moieties can be deprotected by treatment with an excess of neutral hydroxylamine. Since the protecting acetyl groups can be removed without adding disulfide reducing agents like dithiothreitol, disulfides indigenous to the native affinity protein will not be affected. This is an important consideration if disulfides are vital to the binding activity of the affinity protein.

In still another embodiment of the invention, affinity proteins are used which are attached to the liposomal surface through non-covalent, high affinity interactions

such as the biotin-(strept)avidin interaction. For example, biotinylated liposomes may be derivatized with biotinylated proteinaceous affinity components using (strept)avidin as bridging molecule or may be derivatized with a proteinaceous affinity component covalently conjugated with (strept)avidin. It is preferred to employ a long-chain spacer in constructing the biotin-PE derivative to allow sufficient spatial separation from the bilayer surface to accomodate (strept)avidin docking.

V.7. SIGNAL AMPLIFICATION USING ADDITIONAL POLYMERIC CARRIER MOLECULES

The basic assay procedure of the present invention includes binding of analyte (target nucleic acids or amplicons thereof) to immobilized capture oligonucleotides by molecular biological interactions, detection of captured nucleic acids or amplicons thereof by specific binding of affinity liposomes containing encapsulated reporter molecules, e.g. redox mediators, removal of non-bound affinity liposomes, release of reporter molecules encapsulated in specifically bound liposomes, and detection and preferably quantitation of released reporter molecules by means of amperometry or voltammetry using interdigitated array electrodes.

The detection sensitivity achieved by this basic procedure will be sufficient for many applications since affinity liposomes allow excellent detectability of a binding event. Binding of one small affinity liposome to a captured target nucleic acid provides up to 10^5 encapsulated molecules of redox mediators. However, for applications that require higher detection sensitivity, an amplification step may be required. Preferred amplication techniques of this invention are non-enzymatic amplication methods.

In one preferred embodiment of amplification techniques, captured target nucleic acids or amplicons thereof are detected by hydrophilic polymers containing two different affinity components, one for specific binding to captured nucleic acids and the other for binding of affinity liposomes. Thereby, each captured nucleic acid molecule binds a multidude of affinity liposomes since polymers allow covalent attachment of multiple affinity components. Using this technique, the signal may be amplified by one to two orders of magnitude.

Preferred examples of synthetic and natural polymer derivatives are selected from the grupo coprising derivatives of polysaccharides, poly(amino acids), poly(vinyl

alcohols), poly(vinyl pyrrolidinones), poly(acrylic acids), various polyurethanes, polyphosphazenes, and copolymers of these polymers. In a more preferred embodiment, derivatives of dextran are employed as polymeric carrier molecules.

Preferred amplified assay procedures utilizing polymeric carrier systems are those in which captured target nucleic acids or amplicons thereof are detected by dextran polymers containing two types of affinity components. One of the covalently linked affinity components is capable of specifically binding to a specific captured target nucleic acid or its amplicons in a structure restricted manner (e.g., intercalating agents or oligonucleotides). The other covalently linked affinity components are capable of specifically binding multiple affinity liposomes. Subsequently, bound dextran polymers are detected by affinity liposomes containing surface-attached affinity components capable of binding to the affinity components on the dextran polymers. The release of redox mediators encapsulated in specifically bound affinity-liposomes is effected by an increase of the ambient temperature or the addition of liposome-lysing solvents such as organic solvents or detergents. The quantity of released redox mediators is a proportional measure of the amount of target nucleic acids in the specimen.

V.7.1. Affinity systems for binding of affinity liposomes to polymeric carrier molecules

To guarantee efficient binding of affinity liposomes to polymeric carrier molecules, high affinity binding components are preferred. Suitable affinity systems include but are not limited to hapten / anti-hapten antibody affinity systems, enzyme inhibitor / enzyme affinity systems, and the biotin / (strept)avidin affinity system. For example, affinity liposomes containing surface-attached proteinaceous affinity components (e.g., anti-hapten antibodies, enzyme molecules, or (strept)avidin) may be utilized for the detection of polymeric carrier molecules containing the corresponding low molecular weight affinity partner (e.g., hapten molecules, enzyme inhibitors, or biotin residues).

V.7.1.1. Enzyme inhibitor / enzyme affinity systems

In one preferred embodiment, enzyme inhibitors and corresponding enzymes are used for additional affinity binding steps. Several considerations are important for the choice of enzyme inhibitors suitable for use in the present invention. High affinity binding of the inhibitor to the corresponding enzyme is the most important requirement. The overall binding constant (K_{off}/K_{on}) should be in the low nanomolar to picomolar range to guarantee tight binding. Methotrexate

represents one example of such an inhibitor. Methotrexate binds to dihydrofolate reductase (DHFR) with an overall binding constant of 2.1×10^{-10} M.

One preferred approach of increasing the affinity of enzyme inhibitors is the construction of multisubstrate adduct inhibitors (Broom, A.D. J. Med. Chem. 32, 2, 1989). In principle, such inhibitors can be designed for any enzyme that binds two or more substrates simultaneously (cofactors are considered to be substrates in this context). For example, multisubstrate adduct inhibitors for enzymes catalyzing bimolecular reactions can be synthesized by covalent conjugation of both substrates. Typically, the binding affinity of potent multisubstrate adduct inhibitors is 10^3-10^6 times the binding affinity of either substrate. Another preferred approach of increasing the affinity of inhibitor-enzyme interactions is to combine several covalently linked inhibitor molecules with an enzyme complex consisting of two or more copies of the enzyme. Provided the binding sites are in sufficiently close position, simultaneous binding of covalently linked inhibitor molecules is likely to occur. This will increase the apparent affinity of the inhibitor-enzyme interaction significantly.

Further important considerations for the choice of suitable enzyme inhibitors include i) low molecular weight, ii) solubility in aqueous solutions, and iii) the ability of chemical conjugation of the inhibitor to polymers without impairment of the binding affinity. Suitable for use in the invention are water-soluble, small molecular weight inhibitors. In one preferred embodiment, methotrexate (L-4-amino-N^{10}-methylpteroyl-glutamic acid), a water-soluble compound with a molecular weight of 508.5 daltons, is used as inhibitor of DHFR (dihydrofolate reductase). The γ-carboxyl group of the glutamate moiety of this inhibitor can be derivatized without impairing its binding affinity to the enzyme.

Although small molecular weight inhibitors are preferred, high molecular weight inhibitors are also included in this invention. For example, the placental ribonucleae inhibitor (RPI) is a 50kD protein that forms tight complexes with ribonucleases. RPI inhibits RNase A with an extremely low K_i value of 4×10^{-14}M, approaching the affinity of avidin for biotin. Moreover, RPI binds to angiogenin, a blood vessel-inducing protein with 35% sequence homology to pancreatic RNase, with an even lower K_i value of 4×10^{-16} M.

Equally important considerations for the choice of suitable enzyme inhibitor / enzyme affinity systems include i) the molecular weight of the enzyme, ii) the accessibility of purified enzyme, and iii) the ability of chemical conjugation of the enzyme to polymers without impairment of the inhibitor binding affinity. Preferred for use in the invention are enzymes with a low molecular weight which are abundantly present in a variety of organisms. DHFR represents one example of such enzymes. DHFR is found in microorganisms as well as in vertebrates and is easy to purify by affinty chromatography techniques using methotrexate-derivatized matrices. The molecular weight of DHFR of different sources varies between 18 kD and 24 kD. Mammalian DHFR are in general slightly larger (approx. 21 kD) than bacterial DHFR (approx. 18 kD). Furthermore, DHFR has been derivatized with heterobifunctional cross-linking reagents such as LC-SPDP and conjugated to other proteins with good retention of methotrexate binding affinity.

V.7.1.2. Hapten / anti-hapten antibody affinity systems

In another preferred embodiment, haptens and corresponding anti-hapten antibodies are used for additional affinity binding steps. Due to the high affinity of some anti-hapten antibodies, hapten-anti-hapten antibody systems provide sensitive detection reagents. For example, human chromosomes have been probed by hapten-modified cosmid probes with a sensitivity equal to that achieved with biotinylated cosmid probes. The term "antibody" refers to immunoglobulins of any isotype or subclass as well as any fragment (e.g., Fab' of Fv fragments) of the aforementioned. Antibodies of any source are applicable including polyclonal materials obtained from any animal species, monoclonal antibodies from any hybridoma source, and all immunoglobulins (or fragments) generated with the aid of viral, prokaryotic or eukaryotic expression systems. Non-biologic binding molecules such as "molecular imprints" (synthetic polymers with pre-determined specificity for binding or complex formation) are also applicable to the invention. Preferred examples of haptens include but are not limited to digoxin, digoxigenin, dinitrophenol (DNP), trinitrophenol (TNP), biotin, fluorescein, tetramethyl-rhodamin, Texas Red, dansyl residues, lucifer yellow, and Cascade Blue fluorophores. For all of these haptens corresponding high affinity anti-hapten antibodies are commercially available. Especially the very high affinity of anti-fluorescein antibodies makes fluorescein an ideal hapten for various detection schemes. Fluorescein-anti-fluorescein immunoassays have displayed a similar sensitivity as biotin-streptavidin methods in combination with low nonspecific binding.

V.7.1.3. Biotin-(strept)avidin affinity system

In another preferred embodiment, biotin and (strept)avidin are used for additional affinity binding steps. Avidin is a glycoprotein found in egg whites that contains four identical subunits of 16,400 daltons each, giving an intact molecular weight of approximately 66 kD. Each subunit contains one binding site for biotin. The biotin interaction with avidin is among the strongest non-covalent affinities known, exhibiting an affinity constant of about 1.3×10^{-15} M. The tetrameric native structure of avidin is resistant to denaturation under extreme chaotropic conditions. One disadvantage of the use of avidin, however, is its tendency to bind nonspecifically to components other than biotin due to its carbohydrate content and its high pI of about 10. The strong positive charge on the protein causes ionic interactions with more negatively charged molecules such as nucleic acids.

Streptavidin is another biotin binding protein from *Streptomyces avidinii* that can overcome some of the nonspecificities of avidin. Similar to avidin, streptavidin contains four subunits, each with a single biotin binding site. The intact tetrameric protein has a molecular mass of about 60 kD, slightly less than that of avidin. Like avidin, streptavidin is an extremely robust protein that can tolerate a wide range of buffer conditions, pH values, and chemical modification processes without loss of biotin binding activity. The primary structure of streptavidin, however, differs considerably from that of avidin despite the fact that they both bind biotin with similar avidity. This variation in the amino acid sequence results in a much lower pI of 5-6. Moderation in the overall charge of the protein substantially reduces the amount of nonspecific binding due to ionic interaction with other molecules. Of additional significance is the fact that streptavidin is not glycosylated. Thus, for the detection and quantification of nucleic acids streptavidin provides a significant advantage over avidin.

V.7.2. Coupling of affinity components to polymeric carrier molecules

The methods by which the above listed affinity components may be derivatized and covalently coupled to polymers are numerous and well-known in the art. For example, affinity components containing nucleophilic moieties such as a primary amine, a thiol, or a hydroxyl group may be reacted with residues on polymeric carrier molecules that contain electrophilic moieties or have been derivatized with such a moiety. Examples of electrophilic moieties include, but are not limited to alkyl halides, alkyl sulfonates, active esters such as N-hydroxysuccimide esters,

aldehydes, ketones, isothiocyano, maleimido, and carboxylic acid chloride residues. Vice versa, residues on polymeric carrier molecules containing a nucleophilic moiety can be reacted with an electrophilic moiety on the affinity component. Thus, any of a wide range of functional groups on both the affinity components and the polymeric crrier molecules may be utilized for conjugation provided these groups are complementary.

For covalent coupling of proteinaceous affinity components such as enzymes, antibodies, and streptavidin to polymeric carrier molecules, one strategy involves the use of hetero- or homobifunctional cross-linking reagents. For example, protein components may be derivatized with pyridyl disulfide groups (e.g., by reaction with SPDP) and subsequently coupled to sulfhydryl-containing polymeric carrier molecules via disulfide linkages. Alternatively, the introduced sulfhydryl residues may be reduced with disulfide reducing agents to create terminal sulfhydryl groups for coupling to sulfhydryl-reactive polymeric carrier molecules. If SPDP should affect the binding activity of one of these proteins, there are a number of additional cross-linking reagents for coupling via disulfide bonds such as 2-iminothiolane (2-IT) and N-succinimidyl S-acetylthioacetate (SATA). 2-IT reacts with primary amines, instantly incorporating an unprotected sulfhydryl group. SATA also reacts with primary amines, but incorporates a protected sulfhydryl group, which is subsequently deacetylated using neutral hydroxylamine to produce a free sulfhydryl group. Other cross-linkers are available that can be used in different strategies for covalent coupling of proteinaceous affinity components to polymeric carrier molecules. S-(2-Thiopyridyl)-L-cysteine hydrazide (TPCH) and S-(2-thiopyridyl)mercaptopropiono hydrazide (TPMPH) react with the carbohydrate moieties of glycoproteins such as antibodies that have been previously oxidized by mild periodate treatment, thus forming a hydrazone bond between the hydrazide portion of the cross-linking reagent and the periodate-generated aldehydes. TPCH and TPMPH introduce pyridyl disulfide residues which can be used for coupling of the protein components to polymeric carrier molecules as described above. If disulfide bonding is unfavorable, other cross-linking reagents may be used. For example, N-(γ-maleimidobutyryloxy)-succinimide (GMBS) and SMCC (see above) react with primary amines, thereby introducing a maleimide group for coupling to sulfhydryl-containing polymeric carrier molecules via stable thioether linkages. Furthermore, cross-linking reagents may be used which introduce long spacer arms if steric hindrance problems interfere with the binding activity of the covalently coupled affinity component. Thus, there is an abundance of suitable cross-linking reagents which

could be used. Suitable reactions would be well known to one skilled in the art based on the nature of the reactive groups that are available or have been introduced to the molecules and information about the binding site requirements of the affinity components.

The methods by which the above listed low molecular weight affinity components such as inhibitors, haptens, and biotin residues may be derivatized and covalently coupled to polymeric carrier molecules are also numerous and well-known in the art. Several biotin and hapten derivatives are commercially available which provide hydrazide and sulfhydryl groups as well as amine- and sulfhydryl-reactive residues. Examples of low molecular weight affinity components include but are not limited to D-biotinyl--aminocaproic acid-N-hydroxy-succinimide ester and digoxigenin-3-O-methyl-carbonyl-ε-aminocaproic acid-N-hydroxy-succinimide ester for coupling to amine-containing polymeric carrier molecules, and methotrexate-γ-hydrazide (Rosowsky, A., Forsch, R., Uren, J., and Wick, M. J. Med. Chem. 24, 1450, 1981) for coupling to aldehyde-containing polymeric carrier molecules.

V.7.2.1. Coupling of affinity components to dextran carrier molecules

As mentioned above, derivatives of dextran polymers may be employed as polymeric carrier molecules. Dextran is a naturally occurring, mainly a linear polysaccharide consisting of repeating units of D-glucose linked together in glycosidic bonds wherein the carbon-1 of one monomer is attached to the hydroxyl group at the carbon-6 of the next residue. Occasional branch points also may be present in a dextran polymer, occurring as α-1,2, α-1,3, or α-1,4 glycosidic linkages. The monomers contain at least three hydroxyls that may undergo derivatization reactions. This multivalent nature of dextran allows affinity components to be attached at numerous sites along the polymer chain. Soluble dextran polymers of molecular weight of 10,000 - 50,000 have been used in the past as a modifying agent for proteins and other molecules including the application as a carrier of biotin residues and hapten molecules. Furthermore, dextran polymers have been used as a multifunctional linker to cross-link monoclonal antibodies with low molecular weight compounds.

In one preferred embodiment, dextran polymers are used which are oxidized with periodate to produce aldehydes. This procedures results in two aldehyde groups formed per glucose monomer, thus producing a highly reactive, multifunctional

polymer able to couple with numerous amine-containing molecules (Bernstein, A. et al., J. Natl. Cancer Inst. 60, 379, 1978). Polyaldehyde dextran may be conjugated with amine groups by Schiff base formation followed by reductive amination to create stable secondary (or tertiary amine) linkages. Amine-containing affinity components may be coupled to oxidized dextran polymers under mild conditions using sodium cyanoborohydride as reducing agent. The optimal pH for the reductive amination reaction is an alkaline environment between pH 7 and 10.

In another preferred embodiment, dextran derivatives containing carboxy or amine-terminal groups are used for coupling of affinity components. Amine-terminal derivatives may be prepared by coupling diamine compounds such as ethylene diamine or diaminodipropylamine (3,3"-imino*bis*propylamine) in excess to polyaldehyde dextran. Carboxyl-terminal derivatives may be prepared similarly by coupling molecules such as 6-aminocaproic acid or β-alanine to polyaldehyde dextran. Further, reactive alkyl halogen compounds containing a terminal carboxylate group on the other end such as chloroacetic acid or 6-bromohexanoic acidmay be used. The carboxylates may then be aminated with ethylene diamine to form an amine-terminal spacer and further reacted with amine-reactive heterobifunctional reagents to prepare dextran derivatives carrying terminal sulfhydryl residues or sulfhydryl-reactive groups such as pyridyl disulfide, maleimide, or iodoacetyl groups. Such dextran derivatives are used for covalent coupling of affinity components containing free sulfhydryl groups or derivatized with sulfhydryl-reactive residues.

V.8. SIGNAL AMPLIFICATION USING PREFORMED COMPLEXES OF AFFINITY LIPOSOMES

In another specific embodiment of the present invention, preformed complexes of affinity liposomes are utilized for signal amplification. Complexes of affinity liposomes may be prepared from affinity liposomes containing two types of surface-attached affinity components, one (type I) for specific binding to captured nucleic acids or amplicons thereof in a structure restricted manner (e.g., intercalating agents or oligonucleotides) and the other (type II) for complexation of affinity liposomes via bridging molecules providing at least two binding sites for type II affinity components.

In another preferred procedure, polymeric carrier molecules (e.g., derivatives of dextran polymers) containing covalently coupled affinity components are

employed as bridging molecules for the preparation of preformed complexes of affinity liposomes. The application of polymeric carrier molecules for complexation of affinity liposomes may be useful when affinity components containing only a single binding site for the correponding affinity partner are utilized as bridging molecules. The methods by which affinity components may be coupled covalently to polymeric carrier molecules are described in detail in previous sections of the preferred embodiment.

In a further specific embodiment of the present invention, complexes of affinity liposomes are used which are prepared with two types of affinity liposomes containing different affinity components. The affinity components on type I affinity liposomes are capable of specifically binding to a specific captured target nucleic acid or its amplicons in a structure restricted manner (e.g., single-stranded oligonucleotides complementary to single-stranded segments of captured target nucleic acid). The affinity components on type II affinity liposomes are capable of specifically binding to the affinity components o type I affinity liposomes (e.g., single-stranded oligonucleotides complementary to type I single-stranded oligonucleotides).

In specific amplified assay procedures of the invention utilizing preformed complexes of affinity liposomes, captured target nucleic acids or amplicons thereof are detected by specific binding of preformed complexes of affinity liposomes containing surface-attached nucleic acid-reactive affinity components. Subsequent detection and preferably quantitation of reporter molecules encapsulated in specifically bound affinity liposome-complexes is performed as described.

In other specific amplified assay procedures, combinations of polymeric carrier systems and preformed complexes of affinity liposomes are employed for signal amplification. For example, captured target nucleic acids or amplicons thereof may be detected by dextran polymers containing two types of covalently linked affinity components, one being capable of specifically binding to a specific captured target nucleic acid or its amplicons in a structure restricted manner, and the other being capable of specifically binding preformed complexes of affinity liposomes. Subsequent quantitation of redox mediators encapsulated in specifically bound complexes of affinity liposomes is performed as described.

V.8.1. Bridging molecules

Suitable bridging molecules include but are not limited to a) bi- or oligovalent anti-hapten antibodies or fragments thereof, as well as conjugates or fusion constructs thereof; b) enzymes, enzyme conjugates, and fusion constructs of enzymes providing more than one inhibitor-binding site; and c) avidin and streptavidin. Any bridging molecule that provides more than one binding site is useful for this type of amplification methodology.

V.8.1.1. Use of (strept)avidin as bridging molecule

(Strept)avidin may be employed as bridging molecule for complexation of affinity liposomes carrying surface-attached biotin residues and nucleic acid-reactive affinity components such as intercalating agents or oligonucleotides. Since streptavidin contains four biotin binding sites, it represents a preferred multivalent bridging molecule for the synthesis of preformed complexes of affinity liposomes. Multivalent complexes formed by reaction of biotinylated liposomes with (strept)avidin have been shown to increase the detection sensitivity by one to two orders of magnitude.

V.8.1.2. Use of antibodies as bridging molecules

Alternatively, it is possible to use high affinity anti-hapten antibodies or fragments thereof which provide more than one hapten-binding site, as bridging molecules for the synthesis of preformed complexes of affinity liposomes containing surface-attached hapten molecules and surface-attached nucleic acid-reactive components (e.g., intercalating agents or oligonucleotides). For example, intact IgG molecules provide two hapten binding sites and have been applied successfully for the preparation of soluble enzyme / anti-enzyme complexes such as peroxidase / anti-peroxidase (PAP) and alkaline phosphatase / anti-alkaline phosphatase (APAAP) complexes. Alternatively, anti-hapten antibodies of the IgM class providing 10 hapten binding sites per antibody molecule may be employed. In another preferred embodiment, dimers or oligomers of IgG antibodies or hapten-binding fragments thereof are prepared by chemical conjugation methods using long heterobifunctional cross-linking reagents such as LC-SPDP. In still another preferred embodiment, two or more antibody binding regions with specificity for the desired hapten are conjugated by genetic engineering technology and expressed as fusion proteins. Useful constructs of antibody binding regions include but are not limit to single chain fragments of variable antibody regions (scFv) containing on the same polypeptide chain V_H and V_L genes joined with a short linker DNA which codes for example for a flexible $(Gly_4Ser)_3$ peptide (Clackson, T. et al., Nature 352, 624, 1991). For dimeric anti-

hapten scFv fusion proteins, the term 'fusion protein' refers to a genetically engineered protein whose coding region is comprised of the coding region residues of a first anti-hapten scFv molecule fused, in frame, to the coding region residues of a second anti-hapten scFv molecule. For the construction of oligomeric anti-hapten scFv fusion proteins, the dimeric anti-hapten scFv fusion protein construct is extended with coding region residues of additional anti-hapten scFv molecules as described for the dimeric fusion construct. Such constructs may also contain additional flexible oligopeptide linker fused, in frame, to the coding region residues of the scFv molecules.

V.8.1.3. Use of enzymes as bridging molecules

Further, it is possible to use enzyme conjugates providing at least two inhibitor binding sites as bridging molecule for the synthesis of preformed complexes of affinity liposomes containing surface-attached inhibitor molecules and surface-attached nucleic acid-reactive components (e.g., intercalating agents or oligonucleotides). In a more preferred embodiment, dimers and higher oligomers of DHFR prepared by chemical conjugation methods are used as bridiging molecules. Preferred conjugation procedures use heterobifunctional cross-linking reagents containing long spacer molecules between the two functionalities such as LC-SPDP. In another preferred embodiment, two or more DHFR molecules, DHFR mutants, or methotrexate-binding DHFR fragments (all of these referred to as DHFR) are conjugated by genetic engineering technology and expressed as fusion proteins. DHFR from various sources has been cloned and expressed in prokaryotic expression systems since the enzyme consists of a single polypeptide chain containing no disulfide linkages or posttranslational modifications. Due to these characteristics, DHFR represents a useful enzyme for the construction of fusion proteins. For dimeric DHFR fusion proteins, the term 'fusion protein' refers to a genetically engineered protein whose coding region is comprised of the coding region residues of a first DHFR molecule fused, in frame, to the coding region residues of a second DHFR molecule. For the construction of oligomeric DHFR fusion proteins, the dimeric DHFR fusion protein construct is extended with coding region residues of additional DHFR molecules as described for the dimeric fusion construct. Such constructs may also contain flexible oligopeptide linker fused, in frame, to the coding region residues

V.8.2. Synthesis of affinity liposomes suitable for complexation

Affinity liposomes useful for the preparation of preformed complexes contain two types of surface-attached affinity components, one for specific binding to captured

nucleic acids (or to polymeric carrier molecules) and the other for complexation of affinity liposomes (e.g. via bridging systems or via polymeric carrier molecules). The synthesis procedures of such affinity liposomes are identical with those described in previous sections of the present specification. Although two types of lipid derivatives are incorporated into the lipid bilayer construction, the overall molar ratios of derivatized lipid components versus non-derivatized lipid components remain unchanged in preferred lipid compositions. In one preferred embodiment, both affinity components are covalently attached to purified lipid components prior to incorporation into the same lipid bilayer construction. In another preferred embodiment, purified lipid components derivatized with different reactive residues are incorporated into the same lipid bilayer construction and then reacted with the two affinity components carrying different complementary functionalities. In still another preferred embodiment, the activation process and subsequent coupling step of the two affinity components is performed after formation of the intact liposome.

V.9. DETECTION DEVICE

This invention comprises a system that consists of a solid support containing immobilized capture oligonucleotides, affinity liposomes containing reporter molecules, e.g. redox reporter molecules, and an electrochemical sensor. If the electrochemical sensor comprises miniaturized electrodes, this offers effective mass transfer of redox species and the like between the electrodes independent of artificial convection resulting in enhanced signal to noise ratios. In one preferred embodiment, detection devices are further miniaturized by utilization of the microelectrodes as the solid support for immobilization of capture oligonucleotides. For example, sulfhydryl-derivatized capture oligonucleotides may be used for immobilization onto gold microelectrodes. If immobilization techniques are utilized which cannot be performed on microelectrodes, the environment, e.g. the casing of the microelectrodes may be used as solid phase. Utilization of the casing as solid-phase may be an advantage for capturing trace amounts of target nucleic acids since the casing allows immobilization of large quantities of capture oligonucleotides.

VI. SPECIFIC EXAMPLES

VI.1. ACTIVATION AND IMMOBILIZATION OF CAPTURE OLIGONUCLEOTIDES

VI.1.1. Synthesis of 5'-amine derivatives of oligonucleotides

Method A. Covalent attachment of an amine terminal spacer molecule to the 5'-phosphate of oligonucleotides according to method A is performed via formation of a phosphorimidazolide intermediate in a carbodiimide reaction (based on the method of Ghosh, S.S., Kao, P.M., and Kwoh, D.Y. Anal. Biochem. 178, 43, 1989). The formation of a phosphorimidazolide intermediate provides better reactivity towards amine nucleophiles than the carbodiimide phosphosdiester intermediate if carboddimide is used without added imidazole. The carbodiimide phosphodiester intermediate also is shorter-lived in aqueous conditions due to hydrolysis than the imidazolide.

The 5'-phosphate-containing oligonucleotide (7.5 -15 nmol in 7.5 µl) is added to a microfuge tube containing 1.25 mg of the water-soluble carbodiimide EDC (1-ethyl-3-(3-dimethylamino propyl) carbodiimide hydrochloride; Piece Chemical Company, Rockford, IL, USA). Immediately, 5 µl of 0.25 M bis-hydrazide compound (e.g., carbohydrazide or adipic acid dihydrazide) dissolved in 0.1 M imidazole, pH 6, is added. The reaction mixture is vortexed and centrifuged in a microcentrifuge at maximal rpm for 5 min. Thereafter, an additional 20 µl of 0.1 M imidazole, pH 6, is added and the reaction mixture incubated for another 30 min at room temperature. The hydrazide-labeled oligonucleotide is purified by gel filtration on Sephadex G-25 using 10 mM sodium phosphate, 0.15 M NaCl, 10 mM EDTA, pH 7.2.

For derivatization of 5'-phosphate-containing oligonucleotides with diamine spacer molecules (e.g., ethylene diamine or 1,6-diaminohexane) the same experimental procedue is used. The diamine compound is dissolved at a concentration of 0.25 M in 0.1 M imidazole, pH 6, and 5 µl of this soluition is added to the reaction mixture.

Method B. Using method B, the desired oligonucleotide is prepared using automated standard solid-phase phosphoramidite techniques on a scale of about 1 µmol of bound first nucleoside. The solid-phase synthesis protocol includes a) removal of dimethoxytrityl groups with 3% dichloroacetic acid in 1,2-

dichloroethane, b) coupling of the incoming nucleoside 3'-methoxymorpholinophosphite with tetrazole, c) iodine oxidation of the intermediate phosphite to a phosphate, and d) a capping step with acetic anhydride. After synthesis of the required sequence, an extra round of synthesis is carried out using 25 μmol of N-monomethoxytrityl O-methoxydiiso propylaminophosphinyl 3-aminopropan (1) ol (prepared according to Connolly, B.A. Nucleic Acids Res. 15, 3131, 1987) and 60 μmol of tetrazole. Thereafter, the methyl phosphate protecting groups are removed with thiophenol and cleavage from the resin together with deblocking of the base protecting groups is effected with ammonia. The crude monomethoxytrityl protected oligonucleotide is purified by C-18 reverse phase HPLC (flow rate of 1 ml/min) using a gradient of 0.1 M triethylammonium acetate, pH 6.5, containing 5% CH_3CN (buffer A) and 0.1 M triethylammonium acetate, pH 6.5, containing 65% CH_3CN (buffer B) (25% B at t = 0 min ; 75% B at t = 20 min). After removal of the solvent by evaporation, the purified monomethoxytrityl protected oligonucleotide is dissolved in 2 ml of 80% acetic acid and incubated for two hours. Thereafter, the acetic acid is removed by evaporation and the detritylated amino-containing oligonucleotide is is redissolved in a small volume of water.

VI.1.2. Synthesis of 5'-sulfhydryl derivatives of oligonucleotides

Method A. For the derivatization of 5'-phosphate-containing oligonucleotides with a terminal sulfhydryl group according to method A (based on a procedure of Ghosh, S.S. et al., Bioconjugate Chem. 1, 71, 1990), the oligonucleotide (7.5 -15 nmol in 7.5 μl) is added to a microfuge tube containing 1.25 mg of the water-soluble carbodiimide EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; Piece Chemical Company, Rockford, IL, USA). Immediately, 5 μl of 0.25 M cystamine dissolved in 0.1 M imidazole, pH 6, is added. The reaction volume is mixed by vortexing and centrifuged in a microcentrifuge at maximal rpm for 5 min. Thereafter, an additional 20 μl of 0.1 M imidazole, pH 6, is added and the reaction mixture incubated for another 30 min at room temperature. For reduction of the cystamine-derivatized oligonucleotide, 20 μl of 1 M dithiothreitol is added. Thereby, 2-mercaptoethylamine is released from the cystamine modification site and a terminal free sulfhydryl group is created at the 5'-position of the oligonucleotide. After 15 min at room temperature, the sulfhydryl-derivatized oligonucleotide is purified by gel filtration on Sephadex G-25 using 10 mM sodium phosphate, 0.15 M NaCl, 10 mM EDTA, pH 7.2.

Method B. Using method B, the desired oligonucleotide is prepared by automated standard solid-phase phosphoramidite techniques on a scale of about 2.5 μmol of bound first nucleoside. The solid-phase synthesis protocol includes a) removal of dimethoxytrityl groups with 3% dichloroacetic acid in 1,2-dichloroethane, b) coupling of the incoming nucleoside 3'-methoxymorpholinophosphite with tetrazole, c) iodine oxidation of the intermediate phosphite to a phosphate, and d) a capping step with acetic anhydride. After synthesis of the required sequence, an extra round of synthesis is carried out using 25 μmol of an S-trityl-O-methoxymorpholinophosphite derivative of 2-mercaptoethanol (dissolved in 0.3 ml of acetonitile and 0.2 ml of 1,2-dichloroethane), 3-mercaptopropan (1) ol (dissolved in 0.5 ml of acetonitrile), or 6-mercaptohexan (1) ol (dissolved in 0.5 ml of acetonitrile) and 75 μmol of tetrazole (dissolved in 0.5 ml of acetonitrile). The S-trityl-O-methoxymorpholinophosphite derivatives are prepared according to Connolly, B.A., and Rider, P. (Nucleic Acids Res. 13, 4485, 1985). Following coupling, the phosphite intermediate is oxidized by treatment with iodine. Thereafter, the phosphate protecting groups are removed with thiophenolate and cleavage from the resin together with deblocking of the base protecting groups is effected with ammonia. The crude monomethoxytrityl protected oligonucleotide is purified by C-18 reverse phase HPLC (flow rate of 1 ml/min) using a gradient of 0.1 M triethylammonium acetate, pH 6.5, containing 5% CH_3CN (buffer A) and 0.1 M triethylammonium acetate, pH 6.5, containing 65% CH_3CN (buffer B) (10% B at t = 0 min ; 80% B at t = 30 min; 100%B at t = 40 min). The purified S-trityl-containing oligonucleotide in 0.1 M triethylammonium acetate, pH 6.5, is treated with a five-fold molar excess of $AgNO_3$. After 30 min a seven-fold molar excess of dithiothreitol is added and after another 30 min the precipitated Ag^+ salt of dithiothreitol is removed by centrifugation. After reduction of the volume by rotary evaporation, the thiol-containing oligonucleotide is used immediately for further derivatization. Alternatively, the thiol-containing oligonucleotide can be stored frozen at -20 °C for months with no decomposition.

VI.1.3. Pyridyl disulfide modification of 5'-amine-containing oligonucleotides

Oligonucleotides that have been modified with an amine-terminal spacer molecule can be reacted further with the heterobifunctional cross-linking reagent SPDP (N-succinimidyl 3-(2-pyridyldithio)propionate; Pierce Chemical Company, Rockford, IL, USA). Oligonucleotides derivatized with a terminal pyridyl disulfide residue

then can be coupled with sulfhydryl-containing molecules, forming a disulfide bond. Reduction of the terminal pyridyl disulfide residue releases the pyridine-2-thione leaving group and generates a terminal sulhydryl group. This procedure allows conjugation of the 5'-thiolated oligonucleotide to sulfhydryl-reactive derivatives.

First, SPDP is dissolved at a concentration of 6.2 mg/ml in DMF (makes a 20 mM stock solution). The amine-derivatized oligonucleotide is dissolved in 250 µl of 50 mM sodium phosphate, pH 7.5, and mixed with 50 µl of the SPDP solution. After reaction for 1 hour at room temperature, excess reagents are removed from the modified oligonucleotide by gel filtration.

To release the pyridine-2-thione leaving group and form the free sulfhydryl, the oligonucleotide derivative is mixed with 20 µl of 1 M dithiothreitol and incubated for 15 min at room temperature. If present in sufficient quantity, the release of pyridine-2-thione can be monitored by its characteristic absorbance at 343 nm (ε = 8.08 x 10^3 M^{-1} cm^{-1}). The thiolated oligonucleotide is purified from ecxess dithiothreitol by dialysis or gel filtration using 50 mM sodium phosphate, 1 mM EDTA, pH 7.2. The thiolated oligonucleotide is used immediately for further coupling reactions to prevent sulfhydryl oxidation.

VI.1.4. Thioester modification of 5'-amine-containing oligonucleotides

The NHS ester of SATA (Pierce Chemical Company, Rockford, IL, USA) introduces a thioester moiety. The acetyl protecting group can be removed by treatment with neutral hydroxylamine. The resulting terminal sulfhydryl group can be used for subsequent conjugation to thiol-reactive molecules. The advantage of using SATA over disulfide-containing thiolation reagents such as SPDP is that the introduction of sulfhydryl residues does not include the use of a disulfide reducing agent. The pyridiyl dithiol group resulting from an SPDP thiolation must be reduced with a reducing agent such as dithiothreitol to free the sulfhydryl group. With SATA, the sulfhydryl is freed by hydroxylamine, thus eliminating the need for removal of sulfhydryl reductants prior to a conjugation reaction.

First, SATA is dissolved at a concentration of 8 mg/ml in DMF. The amine-derivatized oligonucleotide is dissolved in 250 µl of 50 mM sodium phosphate, pH 7.5, and mixed with 250 µl of the SATA solution. After reaction for 3 hours at 37

°C, excess reagents are removed from the modified oligonucleotide by gel filtration.

To deprotect the thioacetyl group, 100 µl of 50 mM hydroxylamine hydrochloride, 2.5 mM EDTA, pH 7.5, is added and reacted for 2 hours at 37 °C. The sulfhydryl-containing oligonucleotide is used immediately for further reaction with a sulfhydryl-reactive molecule.

VI.1.5. Derivatization of oligonucleotides with an aldehyde function at the 5'-terminus

The cross-linking reagent SFB (succinimidyl *p*-formylbenzoate) can be used to add aldehyde groups to amine-containing oligonucleotides. First, SFB is dissolved at a concentration of 12.35 mg/ml in acetonitrile (makes a 50 mM solution). The amine-derivatized oligonucleotide is dissolved in 250 µl of 50 mM sodium phosphate, pH 7.5, and mixed with 50 µl of the SFB solution. After reaction for 3 hours at room temperature, excess reagents are removed from the modified oligonucleotide by gel filtration.

VI.1.6. Coupling of activated capture oligonucleotides to spacer molecules

To increase the fexibility and, thereby, the reactivity of immobilized capture oligonucleotides, spacer molecules are introduced between the solid support and the immobilized capture oligonucleotides.

Method A. In method A, the heterobifunctional reagents LC-SPDP or sulfo-LC-SPDP (both Pierce Chemical Company, Rockford, IL, USA) are utilized as spacer molecules. The sulfo-NHS form of the cross-linker contains a negatively charged sulfonate group that provides water-solubility to the cross-linker.

LC-SPDP is dissolved at a concentration of 8.5 mg/ml in DMF (makes a 20 mM stock solution). If the water-soluble sulfo-LC-SPDP is used, a stock solution in water is prepared just prior to addition of an aliquot to the reaction, since an aqueous solution of the cross-linker will degrade by hydrolysis of the sulfo-NHS ester. A 10 mM stock solution of sulfo-LC-SPDP is prepared by dissolving 5.2 mg/ml water. The amine-derivatized oligonucleotide is dissolved in 250 µl of 50 mM sodium phosphate, pH 7.5, and mixed with 50 µl of the LC-SPDP solution. If the

water-soluble sulfo-LC-SPDP is used, 100 µl of the sulfo-LC-SPDP solution is added. After reaction for 1 hour at room temperature, excess reagents are removed from the modified oligonucleotide by gel filtration.

Method B. In method B, water-soluble heterobifunctional derivatives of polyethylene glycol (PEG) are utilized as spacer molecules. Heterobifunctional PEG derivatives containing an amine-reactive N-hydroxysuccinimidyl (NHS) moiety and a sulfhydryl-reactive vinylsulfone (VS) moiety are especially useful, since the VS moiety is hydrolytically stable in aqueous media. At pH 7, the VS moiety reacts selectively with sulfhydryl groups. Reaction with amino groups proceeds at higher pH, but is still relatively slow.

NHS-PEG-VS (MW 3400; Shearwater Polymers Europe, Enschede, Netherlands) or NHS-PEG-VS (MW 2000; Shearwater Polymers Europe, Enschede, Netherlands) is dissolved in DMF at a concentration of 10 mM. The amine-derivatized oligonucleotide is dissolved in 250 µl of 50 mM sodium phosphate, pH 7.5, and mixed with 100 µl of the NHS-PEG-VS solution. After reaction for 1 hour at room temperature, excess reagents are removed from the modified oligonucleotide by gel filtration.

VI.1.7. Immobilization of activated capture oligonucleotides

For the immobilization of activated capture oligodeoxynucleotides, a variety of solid support systems providing different reactive residues is available. The examples describe the preparation of some suitable solid support systems and their application for immobilization of activated capture oligodeoxynucleotides. Each of the described procedures can be easily adjusted for the introduction of spacer molecules between the solid support and the immobilized capture oligonucleotides to increase the fexibility and, thereby, the reactivity of immobilized capture oligodeoxynucleotides. Furthermore, each of the described procedures allows to modify the density of immobilized capture oligonucleotides by co-immobilizing hydrophilic compounds capable of blocking immobilization sites for capture oligodeoxynucleotides (e.g., for amine-reactive solid supports: aminoethanol or monomethoxy-poly(ethylene glycol) containing a terminal amino group).

VI.1.7.1. Immobilization of activated capture oligodeoxynucleotides onto mercapto-activated beaded agarose

The preparation of mercapto-activated cross-linked agarose beads is performed as described by Porath, J., and Axen, R. (Meth. Enzymol. 44, 19, 1976). Sepharose 6B (30 g of moist cake) is washed with water, suction-dried, suspended in 24 ml of 1 M NaOH followed by dropwise addition of epichlorohydrin over 15 min at room temperature with stirring (0.75 ml of epichlorohydrin is added for a degree of subsitution of approximately 50 μmol of SH-groups per gram beads; 4.5 ml of epichlorohydrin is added for a degree of subsitution of approximately 700 μmol of SH-groups per gram beads). The suspension is then incubated with continuous stirring for 2 hrs at 60 °C. The product is washed with water and with 0.5 M sodium phosphate buffer, pH 6.25. After suction-drying and resuspension in 30 ml of 0.5 M sodium phosphate buffer, pH 6.25, 30 ml of 2 M sodium thiosulfate are added to the beads, followed by stirring for 6 hrs at room temperature. The alkyl thiosulfate ester obtained is washed with water, suspended in 60 ml of 0.1 M sodium bicarbonate, and reduced by the addition of 50 ml of dithiothreitol (8 mg/ml) containing 1 mM EDTA. The resulting mercapto beads are washed with 300 ml of 0.1 M sodium bicarbonate, 1.0 M NaCl, 1 mM EDTA, and then with 100 ml of 1 mM EDTA. The mercapto beads are stored in 10 mM deaerated sodium acetate, 1 mM EDTA, pH 4.

Prior to coupling of 5'-pyridyl disulfide derivatized oligodeoxynucleotides, the mercapto-activated cross-linked agarose beads are filtered and washed quickly with 50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA, pH 7.5. Thereafter, the cake is suspended in 50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA, pH 7.5, containing the 5'-pyridyl disulfide-derivatized oligodeoxynucleotide to be immobilized. After 20 hrs at room temperature with gentle agitation, the residue is filtered and washed with 50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA, pH 7.5, and with distilled water. Excess active groups can be removed by reaction with iodoacetate.

VI.1.7.2. Immobilization of activated capture oligodeoxynucleotides onto pyridyl disulfide- activated beaded agarose

Mercapto-activated cross-linked agarose beads (section VI.1.7.1) are washed with water and a solution of 50% acetone-water. Thereafter, the beads are resuspended in 50% aqueous acetone and mixed with 100 mg 2,2'-dipyridyl disulfide dissolved in 50% aqueous acetone. After 30 min at room temperature, the beads are washed with 50% aqueous acetone containing 1 mM EDTA.

Prior to coupling of 5'-sulfhydryl-derivatized oligodeoxynucleotides, the pyridyl disulfide-activated cross-linked agarose beads are filtered and washed with 50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA, pH 7.5. Thereafter, the cake is suspended in 50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA, pH 7.5, containing the 5'-sulfhydryl-derivatized oligodeoxynucleotide to be immobilized. After 20 hrs at room temperature with gentle agitation, the residue is filtered and washed with 50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA, pH 7.5, and with distilled water.

VI.1.7.3. Immobilization of activated capture oligodeoxynucleotides onto carbonyldiimidazole-activated beaded agarose

The preparation of carbonyldiimidazole (CDI)-activated cross-linked beaded agarose is performed as described by Wilchek, M., et al., (Meth. Enzyol. 104, 3, 1984). Cross-linked Sepharose 6B (3 g of moist cake) is washed sequentially with 20 ml each of water, dioxane-water (3: 7), dioxane-water (7: 3), and dioxane and ist suspended in 5 ml of dioxane. 1,1'-Carbonyldiimidazole (120 mg) is added, and the suspension is shaken at room temperature. After 15 min, the suspension is washed with 100 ml dioxane and used immediately. Alternatively, the matrix may be stored in dioxane under anhydrous conditions.

Prior to coupling of 5'-amine derivatized oligodeoxynucleotides, the CDI-activated cross-linked agarose beads are filtered and washed quickly with 0.1 M sodium bicarbonate, pH 8.5. Thereafter, the moist beads are resuspended in 0.1 M sodium bicarbonate, pH 8.5, containing the 5'-amine-oligodeoxynucleotide derivative to be immobilized. After 20 hrs at 4 °C with gentle agitation, the residue is filtered and washed with 0.1 M $NaHCO_3$ and with distilled water. Excess active groups are removed by reaction for 1 hr at room temperature with either 0.1 M NH_4OH or 0.1 M ethanolamine at pH 9.

VI.1.7.4. Immobilization of activated capture oligodeoxynucleotides onto tresyl- or tosyl-activated beaded agarose

The preparation of tresylated or tosylated cross-linked beaded agarose is performed as described by Nilsson, K., and Mosbach, K. (Meth. Enzymol. 104, 56, 1984). Sepharose 4B or CL-6B (10 g, wet) is washed successively with 100 ml of each of the following: 30: 70 and 70: 30 of acetone: water (v/v), twice with acetone, and three times with dry acetone (dried with a molecular sieve overnight) using 1 liter of acetone per 35 g of Sepharose 4B. The gel is then transferred to a

dried beaker containing 3 ml of dry acetone and 150 µl of dry pyridine (dried with a molecular sieve). During magnetic stirring, 100 µl of tresyl chloride (2,2,2-trifluoroethanesulfonyl chloride) (Fluka AG, Buchs, Switzerland) is added dropwise. After 10 min at room temperature, the gel is washed twice with 100 ml of each of the following: acetone, 30: 70 of 5 mM HCl: acetone, 50: 50 of 5 mM HCl: acetone, 70: 30 of 5 mM HCl: acetone, and 1 mM HCl. For tosyl-activation, the beaker contains 0.6 g of tosyl chloride (p-toluenesulfonyl chloride) dissolved in 3 ml of dry acetone. After addition of 1 ml of dry pyridine (dried with a molecular sieve), the reaction is continued for 1 hr at room temperature with continuous magnetic stirring. The activated gel is washed as described for the tresylated gel. The tresylated and the tosylated cross-linked agarose beads are stored at 4 °C until used. The reactivity of Sepharose tresyl groups is very high, allowing a 75 to 100% coupling yield of thiol- or amino-containing molecules within 1 hr at pH 7.5 in the cold. Thiols and primary amino groups are the most reactive nucleophiles with sulfonate esters on gels, thiols showing the highest reactivity.

Prior to coupling of 5'-amine derivatized oligodeoxynucleotides, the tresyl-activated cross-linked agarose beads are washed quickly with cold 0.2 M sodium phosphate, 0.5 M NaCl, pH 8.2. Thereafter, the moist agarose beads (1 g) are resuspended in 1 ml of 0.2 M sodium phosphate, 0.5 M NaCl, pH 8.2, containing the 5'-amine-oligodeoxynucleotide derivative to be immobilized. After 20 hrs at 4 °C with gentle agitation, the gel is treated with 0.2 M Tris-HCl, pH 8.5, for 5 hrs at room temperature, and washed with a) 0.2 M sodium acetate, 0.5 M NaCl, pH 3.5, b) 0.5 M NaCl, and c) distilled water.

Prior to coupling of 5'-amine derivatized oligodeoxynucleotides, the tosyl-activated cross-linked agarose beads are washed quickly with cold 0.25 M $NaHCO_3$ at pH 10.5. Sepharose tosyl groups require a pH of 9 to 10.5 for efficient coupling of amine-containing molecules. Thereafter, the moist agarose beads (0.7 g) are resuspended in 1 ml of 0.25 M $NaHCO_3$, pH 10.5, containing the 5'-amine-oligodeoxynucleotide derivative to be immobilized. After 20 hrs at 40 °C with gentle agitation, the gel is treated with 0.8 M mercaptoethanol, pH 10, for 15 hrs at 40 °C, and washed with a) 0.2 M sodium acetate, 0.5 M NaCl, pH 3.5, b) 0.5 M NaCl, and c) distilled water.

VI.1.7.5. Immobilization of activated capture oligodeoxynucleotides onto sulfhydryl-containing polyacrylhydrazidoagarose (PAHOS)

Sepharose beads derivatized with linear polyacrylic hydrazide (PAHOS) provide properties of Sepharose and polyacrylamide gels. The preparation of PAHOS is performed as described by by Wilchek, M., Miron, T., and Kohn, J. (Meth. Enzyol. 104, 3, 1984). Washed Sepharose 4B (10 g) is suspended in 30 ml of freshly prepared 0.25 M sodium periodate. The suspension is slowly stirred at room temperature for 3 hrs in the dark. The oxidized Sepharose is then washed with cold and resuspended in three volumes of aqueous polyacrylhydrazide solution (0.1 - 0.5%) prepared from polymethylacrylate and hydrazine hydrate as described (Wilchek, M., and Miron, T. Meth. Enzymol. 34, 72, 1974). After slow stirring for 16 hrs in the dark at room temperature, the beads are washed extensively with 0.1 M NaCl, and then reduced with 0.3 M sodium borohydride in 0.5 M Tris-HCl, pH 8, for 3 hrs at room temperature. The reduced gel is washed with water on a sintered-glass funnel and stored at 4 °C.

Sulfhydryl groups are introduced by treating the gel with N-acetylhomocysteine thiolactone. The thiolactone (1 g) is added to a cold suspension of 10 ml of PAHOS beads in 20 ml of 1 M $NaCO_3$. After slow stirring for 16 hrs at 4 °C, the product is washed extensively with water and 0.1 M NaCl.

Prior to coupling of 5'-pyridyl disulfide derivatized oligodeoxynucleotides, the sulfhydryl-activated PAHOS beads are washed quickly with 50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA, pH 7.5. Thereafter, the beads are resuspended in 50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA, pH 7.5, containing the 5'-pyridyl disulfide-derivatized oligodeoxynucleotide to be immobilized. After incubation for 20 hrs at room temperature with gentle agitation, the beads are washed with 50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA, pH 7.5, and with distilled water. Excess active groups can be removed by reaction with iodoacetate.

VI.1.7.6. Immobilization of activated capture oligodeoxynucleotides onto tresyl-activated silica

Porous glass spheres (10 μm diameter, 500 Å pore diamter) (LiChrosphere Si, Altex Scientific, Inc., Berkeley, CA, USA) are treated with hot chromic acid cleaning solution follwed by rinsing with 100 ml per 1 g porous glass spheres of hot 1 M HNO_3 and water. Thereafter, the cleaned porous glass spheres are suspended in a 10% aqueous solution of glycidoxypropyl trimethoxysilane, degassed with ultrasonic vibration for 10 min, and kept for 2 hrs at 90 °C, during

which time the pH is maintained at 3.0 with 1 M HCl. the glass sheres are then collected on a medium-porosity glass frit, rinsed with water and dried overnight at 105 °C in vacuo.

The preparation of tresylated porous glass spheres coated with a hydrophilic layer of glycerylpropyl groups is performed as described by Nilsson, K., and Mosbach, K. (Meth. Enzymol. 104, 56, 1984). The dried porous glass spheres (2 g) is washed three times with 50 ml each of dry acetone (dried with a molecular sieve overnight). The spheres are then transferred to a dried beaker containing 2.5 ml of dry acetone and 130 µl of dry pyridine (dried with a molecular sieve). During magnetic stirring, 90 µl of tresyl chloride (2,2,2-trifluoroethanesulfonyl chloride) (Fluka AG, Buchs, Switzerland) is added dropwise. After 15 min at 0 °C, the spheres are washed twice with 50 ml of each of the following: acetone, 30: 70 of 5 mM HCl: acetone, 50: 50 of 5 mM HCl: acetone, 70: 30 of 5 mM HCl: acetone, and 1 mM HCl. For storage, the tresylated porous glass spheres are washed with water, 50: 50 (v/v) water: acetone, and acetone and dried.

For coupling of 5'-amine derivatized oligodeoxynucleotides, the tresylated porous glass spheres are resuspended in 1 ml of 0.2 M sodium phosphate, 0.5 M NaCl, pH 8.2, containing the 5'-amine-oligodeoxynucleotide derivative to be immobilized. After 20 hrs at 4 °C with gentle agitation, the spheres are treated with 0.2 M Tris-HCl, pH 8.5, for 5 hrs at room temperature, and washed with a) 0.2 M sodium acetate, 0.5 M NaCl, pH 3.5, b) 0.5 M NaCl, and c) distilled water.

VI.1.7.7. Immobilization of activated capture oligodeoxynucleotides onto aldehyde-activated polyester film

The polyester film is a polymer of glycerol and terephthalic acid (Gareware Chemical Co., India) which on partial acid hydrolysis and periodate oxidation provides aldehyde groups (Sarkar, M., and Mandal, C. J. Immunol. Meth. 83, 55, 1985). For partial hydrolysis, the film is hydrolyzed with 1 M H_2SO_4 for 6 hrs at 100 °C, washed with distilled water, and then treated with 0.1 M $NaIO_4$ for 24 hrs at 30 °C. After elimination of excess periodate by the addition of 0.1 M ethylene glycol, the film is washed with distilled water and stored at 4 oC in 50 mM sodium phosphate, 150 mM NaCl, pH 7.5.

For coupling of 5'-hydrazide derivatized oligodeoxynucleotides, the aldehyde-activated polyester film is incubated with the 5'-hydrazide-oligodeoxynucleotide

derivative to be immobilized in 50 mM M sodium phosphate, 150 M NaCl, pH 7.5. After 16 hrs at room temperature with gentle agitation, the film is washed with 50 mM M sodium phosphate, 150 M NaCl, pH 7.5. To reduce the hydrazone bonds to more stable linkages, the film may be further incubated for 1 hr in the presence of 15 mM sodium cyanoborohydride.

VI.2. SELECTION OF SUITABLE REDOX MEDIATORS

Closely spaced interdigitated array (IDA) microelectrodes are utilized to select suitable redox mediators as described by Wollenberger, U., Paeschke, M., and Hintsche, R. (Analyst 119, 1245, 1994). The IDA electrodes consist of four pairs of microbands arranged on one silicon chip. Each finger electrode is 900 μm long and 3.2 μm wide. The gaps between the finger electrodes are 0.8 μm. The silicon chip is encapsulated by a 400 nm thick plasma-enhanced chemical vapour deposition (PECVD) SiO_2 layer, excluding the electrode area and the connecting pads. The chip sensor is electrically connected by means of gold wires bonded to the pads. These bonds are covered with silicon rubber resin.

Table II

Mediator	E_{anodic} [mV]	$E_{cathodic}$ [mV]	Collection Efficiency	Detection limit [nM]
p-aminophenol	350	- 150	0.85	5 - 10
o-hydroquinone	600	- 200	0.92	5 - 10
o-benzoquinone	600	- 200	0.92	5 - 10
dopamine	800	- 100	0.88	20
aderenalin	400	- 50	0.86	50
$Fe(CN)_6^{4-}$	350	- 120	0.98	30
Ferrocene dicarboxylic acid	600	0	0.93	50
Ferrocene lysine	600	0	0.91	5 - 10
Polyethylene glycol ferrocene	400	100	0.94	
Gentisyl aldehyde	650	- 50	0.83	50
Osmiumbipyridyl derivatized with carboxyl groups	0	- 400	0.99	5

Osmiumbipyridyl derivatized with phenyl residues	900	500	0.98	30

Electrochemical measurements are carried out at 25 °C in 66 mM potassium phosphate / sodium phosphate, 100 mM KCl, pH 7.0, using an Ag-AgCl reference electrode (Bioanalytical Systems, West Lafayette, IN, USA). A custom-made multipotentiostat is used to control the potential of the electrodes independently. The current response of the individual electrodes is separately preamplified after current-to-voltage conversion and sent to a data acquisition board. Cyclic voltammograms are ontained with an Auto-Lab PSTAT 10 electrochemical analyzer (ECO Chemie, Utrecht, The Netherlands). In order to define the optimum potentials for oxidation and reduction of suitable mediators, cyclic and hydrodynamic voltammograms are recorded. Results are summarized in the table shown above.

VI.3. DERIVATIZATION OF IINTERCALATING AGENTS WITH REACTIVE RESIDUES

VI.3.1. Derivatization of the daunosamine moiety of daunorubicin with 2-iminothiolane

The cyclic imidoester 2-iminothiolane reacts with amines to form a stable, positively charged linkage, while leaving a sulfhydryl group available for further coupling (Jue, R. et al., Biochemistry 17, 5399, 1978). Using this heterobifunctional reagent, the positive charge of the original amine of the daunosamine moiety is preserved and can bind electrostatically to the negatively charged phosphate groups of the DNA.

Daunorubicin hydrochloride (Sigma, St. Louis, MO, USA) is dissolved in dry DMF at a concentration of 4.5 mg/ml and treated with an excess of silver carbonate (Pietersz, G.A. et al., C. In: Antibody-mediated delivery systems. (J.D. Rodwell, ed.) pp. 25-53, Marcel Dekker, New York, 1988). After shaking for 15 min, the mixture is centrifuged at 450 x g for 10 min. The supernatant is decanted and a slight molar excess of 2-iminothiolane (Pierce Chemical Company, Rockford, IL, USA) in DMF is added. After incubation of the reaction mixture for 3 hours at room temperature with stirring, DMF is evaporated and the residue dissolved in dichloromethane. Diethyl ether is added until precipitation is completed. The precipitate is washed several times with diethyl ether/dichloromethane (2:1) and

finally dried and stored at 4 °C in the dark. The purity of 4-mercaptobutyrimidate-derivatized daunorubicin is analyzed by TLC using silica gel 60 F_{254} plates (Merck, Germany) and n-butanol: acetic acid: water at a ratio of 4: 1: 5 as solvent.

VI.3.2. Derivatization of the daunosamine moiety of daunorubicin with LC-SPDP

The long-chain (LC) version of SPDP provides an additional 6-aminohexanoate spacer group as compared to SPDP. As a consequence, LC-SPDP increases the flexibility of daunorubcin when conjugated to liposomal surfaces and reduces thereby potential steric hindrance problems.

Daunorubicin hydrochloride (Sigma, St. Louis, MO, USA) is dissolved in dry DMF at a concentration of 4.5 mg/ml and treated with an excess of silver carbonate (Pietersz, G.A. et al., In: Antibody-mediated delivery systems. (J.D. Rodwell, ed.) pp. 25-53, Marcel Dekker, New York, 1988). After shaking for 15 min, the mixture is centrifuged at 450 x g for 10 min. The supernatant is decanted and a slight molar excess of LC-SPDP (Pierce Chemical Company, Rockford, IL, USA) in DMF is added. After incubation of the reaction mixture for 3 hours at room temperature with stirring, DMF is evaporated and the residue dissolved in dichloromethane. Diethyl ether is added until precipitation is completed. The precipitate is washed several times with diethyl ether/dichloromethane (2:1) and finally dried and stored at 4 °C in the dark. The purity of LC-SPDP-derivatized daunorubicin is analyzed by TLC using silica gel 60 F_{254} plates (Merck, Germany) and n-butanol: acetic acid: water at a ratio of 4: 1: 5 as solvent.

VI.3.3. Derivatization of the daunosamine moiety of daunorubicin with SIAXX

Since SIAXX contains two aminohexanoate spacer groups, conjugates prepared with this reagent are connected by a spacer arm containing 16 atoms. Thus, SIAXX provides high flexibility to surface-attached daunorubicin molecules.

Daunorubicin hydrochloride (Sigma, St. Louis, MO, USA) is dissolved in dry DMF at a concentration of 4.5 mg/ml and treated with an excess of silver carbonate (Pietersz, G.A. et al., In: Antibody-mediated delivery systems. (.D. Rodwell, ed.) pp. 25-53, Marcel Dekker, New York, 1988). After shaking for 15 min, the mixture is centrifuged at 450 x g for 10 min. The supernatant is decanted and a slight molar excess of SIAXX (Molecular Probes, Eugene, OR, USA) in DMF is added. After incubation of the reaction mixture for 3 hours at room temperature with

stirring, DMF is evaporated and the residue dissolved in dichloromethane. Diethyl ether is added until precipitation is completed. The precipitate is washed several times with diethyl ether/dichloromethane (2:1) and finally dried and stored at 4 °C in the dark. The purity of SIAXX-derivatized daunorubicin is analyzed by TLC using silica gel 60 F_{254} plates (Merck, Germany) and n-butanol: acetic acid: water at a ratio of 4: 1: 5 as solvent.

VI.3.4. Derivatization of the daunosamine moiety of daunorubicin with NHS-PEG-VS

NHS-PEG-VS, heterobifunctional poly(ethylene glycol) derivatives containing an amine-reactive N-hydroxysuccinimidyl (NHS) moiety and a sulfhydryl-reactive vinylsulfone (VS) moiety, are commercially available from Shearwater Polymers Europe (Enschede, The Netherlands). PEG-spacer molecules provide water-solubility and a high degree of flexibility.

Daunorubicin hydrochloride (Sigma, St. Louis, MO, USA) is dissolved in dry DMF at a concentration of 4.5 mg/ml and mixed with a slight molar excess of NHS-PG-VS (MW 2 kD or 3.4 kD) dissolved in DMF at a concentration of 10 mM. After incubation of the reaction mixture for 3 hours at room temperature with stirring, DMF is evaporated and the residue dissolved in dichloromethane. Diethyl ether is added until precipitation is completed. The precipitate is washed several times with diethyl ether/dichloromethane (2:1) and finally dried and stored at 4 °C in the dark. The purity of VS-PG-derivatized daunorubicin is analyzed by TLC using silica gel 60 F_{254} plates (Merck, Germany) and n-butanol: acetic acid: water at a ratio of 4: 1: 5 as solvent.

VI.3.5. Derivation of the N^2-position of actinomycin D with 1,3-propane diamine

Derivatization of the N^2-position of actinomycin D with 1,3-propanediamine is performed in three steps including the synthesis of 2-deamino-2-hydroxyactinomycin D, 2-deamino-2-chloroactinomycin D (both according to Moore, S. et al, J. Med. Chem. 18, 1098, 1975), and N^2-(3'-aminopropyl) actinomycin D (according to Sengupta, S.K. et al., J. Med. Chem. 24, 1052, 1981).

a) Synthesis of 2-deamino-2-hydroxyactinomycin D. A solution of actinomycin D (1.2 g) in 10% HCl (300 ml) is heated for 4.5 hr at 60°C. On cooling, the solution is extracted with chloroform (3 x 200 ml). The chloroform solution is washed twice

with water and saline, dried, and evaporated. The product is chromatographed on Sephadex LH-20 using 95% ethanol as eluent. The purity of 2-deamino-2-hydroxy actinomycin D is confirmed by TLC using silica gel plates and *sec*-BuOH: HCOOH: H_2O (75: 13.5: 11.5) as solvent system.

b) Synthesis of 2-deamino-2-chloroactinomycin D. 2-Deamino-2-hydroxy actinomycin D is dissolved in dry benzene (50 ml). First, chloranil (297 mg) is added, followed by freshly destilled thionyl chloride (8.8 ml). The reaction mixture is refluxed under anhydrous conditions for 25 min. After cooling, the solution is evaporated to dryness and reevaporated several times from benzene. The product is precipitated from benzene with cold hexane and separated by centrifugation. The purity of 2-deamino-2-chloroactinomycin D is confirmed by TLC using silica gel plates and *sec*-BuOH: HCOOH: H_2O (75: 13.5: 11.5) or EtOAc: acetone (2: 1) as solvent system.

c) Synthesis of N^2-(3'-aminopropyl)actinomycin D. 2-Deamino-2-chloro actinomycin D (11 mg) is dissolved in methylene chloride (4 ml) and mixed with 100 µl (140 equiv) of 1,3-propanediamine (over 99.9% pure). After stirring for 3 hr at 45 -50°C under N_2, the reaction mixture is diluted with 50 ml methylene chloride and extracted with water (3 x 10 ml). Thereafter, the organic solvent is removed by evaporation. The purity of N^2-(3'-aminopropyl)actinomycin D is confirmed by TLC using silica gel plates and EtOAc: acetone (2: 1) as solvent system.

VI.3.6. Derivatization of N^2-(3'-aminopropyl) actinomycin D with LC-SPDP

The long-chain (LC) version of SPDP provides a propionamidohexanoate spacer group which increases the flexibility of actinomycin D when conjugated to liposomal surfaces.

N^2-(3'-Aminopropyl)actinomycin D is dissoved in dry DMF and mixed with a slight molar excess of LC-SPDP (Pierce Chemical Company, Rockford, IL, USA) in DMF. After incubation of the reaction mixture for 3 hours at room temperature with stirring, DMF is evaporated and the residue dissolved in dichloromethane. Diethyl ether is added until precipitation is completed. The precipitate is washed several times with diethyl ether/dichloromethane (2:1) and finally dried and stored at 4 °C in the dark. The purity of SIAXX-derivatized daunorubicin is analyzed by

TLC using silica gel 60 F_{254} plates (Merck, Germany) and n-butanol: acetic acid: water at a ratio of 4: 1: 5 as solvent.

VI.3.7. Derivatization of N^2-(3'-aminopropyl) actinomycin D with SIAXX

SIAXX contains two aminohexanoate spacer groups which provide high flexibility to surface-attached actinomycin D molecules.

N^2-(3'-Aminopropyl)actinomycin D is dissoved in dry DMF and mixed with a slight molar excess of SIAXX (Molecular Probes, Eugene, OR, USA) in DMF is added. After incubation of the reaction mixture for 3 hours at room temperature with stirring, DMF is evaporated and the residue dissolved in dichloromethane. Diethyl ether is added until precipitation is completed. The precipitate is washed several times with diethyl ether/dichloromethane (2:1) and finally dried and stored at 4 °C in the dark. The purity of SIAXX-derivatized daunorubicin is analyzed by TLC using silica gel 60 F_{254} plates (Merck, Germany) and n-butanol: acetic acid: water at a ratio of 4: 1: 5 as solvent.

VI.3.8. Derivatization of N^2-(3'-aminopropyl) actinomycin D with NHS-PEG-VS

N^2-(3'-Aminopropyl)actinomycin D is dissolved in dry DMF and mixed with a slight molar excess of NHS-PG-VS (Shearwater Polymers Europe, Enschede, The Netherlands, MW 2 kD or 3.4 kD) dissolved in DMF at a concentration of 10 mM. After incubation of the reaction mixture for 3 hours at room temperature with stirring, DMF is evaporated and the residue dissolved in dichloromethane. Diethyl ether is added until precipitation is completed. The precipitate is washed several times with diethyl ether/dichloromethane (2:1) and finally dried and stored at 4 °C in the dark. The purity of VS-PG-derivatized daunorubicin is analyzed by TLC using silica gel 60 F_{254} plates (Merck, Germany) and n-butanol: acetic acid: water at a ratio of 4: 1: 5 as solvent.

VI.4. DERIVATIZATION OF OLIGONUCLEOTIDES WITH REACTIVE RESIDUES FOR ATTACHMENT TO AFFINITY LIPOSOMES

VI.4.1. Synthesis of 5'-amine derivatives of oligonucleotides

Method A. Covalent attachment of an amine terminal spacer molecule to the 5'-phosphate of oligonucleotides according to method A is performed via formation of a phosphorimidazolide intermediate in a carbodiimide reaction (based on the

method of Ghosh, S.S., et al., Anal. Biochem. 178, 43, 1989). The formation of a phosphorimidazolide intermediate provides better reactivity towards amine nucleophiles than the carbodiimide phosphosdiester intermediate if carboddimide is used without added imidazole. The carbodiimide phosphodiester intermediate also is shorter-lived in aqueous conditions due to hydrolysis than the imidazolide.

The 5'-phosphate-containing oligonucleotide (7.5 -15 nmol in 7.5 μl) is added to a microfuge tube containing 1.25 mg of the water-soluble carbodiimide EDC (1-ethyl-3-(3-dimethylamino propyl) carbodiimide hydrochloride; Piece Chemical Company, Rockford, IL, USA). Immediately, 5 μl of 0.25 M bis-hydrazide compound (e.g., carbohydrazide or adipic acid dihydrazide) dissolved in 0.1 M imidazole, pH 6, is added. The reaction mixture is vortexed and centrifuged in a microcentrifuge at maximal rpm for 5 min. Thereafter, an additional 20 μl of 0.1 M imidazole, pH 6, is added and the reaction mixture incubated for another 30 min at room temperature. The hydrazide-labeled oligonucleotide is purified by gel filtration on Sephadex G-25 using 10 mM sodium phosphate, 0.15 M NaCl, 10 mM EDTA, pH 7.2.

For derivatization of 5'-phosphate-containing oligonucleotides with diamine spacer molecules (e.g., ethylene diamine or 1,6-diaminohexane) the same experimental procedue is used. The diamine compound is dissolved at a concentration of 0.25 M in 0.1 M imidazole, pH 6, and 5 μl of this soluition is added to the reaction mixture.

Method B. Using method B, the desired oligonucleotide is prepared using automated standard solid-phase phosphoramidite techniques on a scale of about 1 μmol of bound first nucleoside. The solid-phase synthesis protocol includes a) removal of dimethoxytrityl groups with 3% dichloroacetic acid in 1,2-dichloroethane, b) coupling of the incoming nucleoside 3'-methoxymorpholinophosphite with tetrazole, c) iodine oxidation of the intermediate phosphite to a phosphate, and d) a capping step with acetic anhydride. After synthesis of the required sequence, an extra round of synthesis is carried out using 25 μmol of N-monomethoxytrityl O-methoxydiiso propylaminophosphinyl 3-aminopropan (1) ol (prepared according to Connolly, B.A. Nucleic Acids Res. 15, 3131, 1987) and 60 μmol of tetrazole. Thereafter, the methyl phosphate protecting groups are removed with thiophenol and cleavage from the resin

together with deblocking of the base protecting groups is effected with ammonia. The crude monomethoxytrityl protected oligonucleotide is purified by C-18 reverse phase HPLC (flow rate of 1 ml/min) using a gradient of 0.1 M triethylammonium acetate, pH 6.5, containing 5% CH_3CN (buffer A) and 0.1 M triethylammonium acetate, pH 6.5, containing 65% CH_3CN (buffer B) (25% B at t = 0 min ; 75% B at t = 20 min). After removal of the solvent by evaporation, the purified monomethoxytrityl protected oligonucleotide is dissolved in 2 ml of 80% acetic acid and incubated for two hours. Thereafter, the acetic acid is removed by evaporation and the detritylated amino-containing oligonucleotide is is redissolved in a small volume of water.

VI.4.2. Synthesis of 5'-sulfhydryl derivatives of oligonucleotides

Method A. For the derivatization of 5'-phosphate-containing oligonucleotides with a terminal sulfhydryl group according to method A (based on a procedure of Ghosh, S.S. et al., Kao, Bioconjugate Chem. 1, 71, 1990), the oligonucleotide (7.5 -15 nmol in 7.5 µl) is added to a microfuge tube containing 1.25 mg of the water-soluble carbodiimide EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; Pierce Chemical Company, Rockford, IL, USA). Immediately, 5 µl of 0.25 M cystamine dissolved in 0.1 M imidazole, pH 6, is added. The reaction volume is mixed by vortexing and centrifuged in a microcentrifuge at maximal rpm for 5 min. Thereafter, an additional 20 µl of 0.1 M imidazole, pH 6, is added and the reaction mixture incubated for another 30 min at room temperature. For reduction of the cystamine-derivatized oligonucleotide, 20 µl of 1 M dithiothreitol is added. Thereby, 2-mercaptoethylamine is released from the cystamine modification site and a terminal free sulfhydryl group is created at the 5'-position of the oligonucleotide. After 15 min at room temperature, the sulfhydryl-derivatized oligonucleotide is purified by gel filtration on Sephadex G-25 using 10 mM sodium phosphate, 0.15 M NaCl, 10 mM EDTA, pH 7.2.

Method B. Using method B, the desired oligonucleotide is prepared by automated standard solid-phase phosphoramidite techniques on a scale of about 2.5 µmol of bound first nucleoside. The solid-phase synthesis protocol includes a) removal of dimethoxytrityl groups with 3% dichloroacetic acid in 1,2-dichloroethane, b) coupling of the incoming nucleoside 3'-methoxymorpholinophosphite with tetrazole, c) iodine oxidation of the intermediate phosphite to a phosphate, and d) a capping step with acetic anhydride. After synthesis of the required sequence,

an extra round of synthesis is carried out using 25 μmol of an S-trityl-O-methoxymorpholinophosphite derivative of 2-mercaptoethanol (dissolved in 0.3 ml of acetonitile and 0.2 ml of 1,2-dichloroethane), 3-mercaptopropan (1) ol (dissolved in 0.5 ml of acetonitrile), or 6-mercaptohexan (1) ol (dissolved in 0.5 ml of acetonitrile) and 75 μmol of tetrazole (dissolved in 0.5 ml of acetonitrile). The S-trityl-O-methoxymorpholinophosphite derivatives are prepared according to Connolly, B.A., and Rider, P. (Nucleic Acids Res. 13, 4485, 1985). Following coupling, the phosphite intermediate is oxidized by treatment with iodine. Thereafter, the phosphate protecting groups are removed with thiophenolate and cleavage from the resin together with deblocking of the base protecting groups is effected with ammonia. The crude monomethoxytrityl protected oligonucleotide is purified by C-18 reverse phase HPLC (flow rate of 1 ml/min) using a gradient of 0.1 M triethylammonium acetate, pH 6.5, containing 5% CH_3CN (buffer A) and 0.1 M triethylammonium acetate, pH 6.5, containing 65% CH_3CN (buffer B) (10% B at t = 0 min ; 80% B at t = 30 min; 100%B at t = 40 min). The purified S-trityl-containing oligonucleotide in 0.1 M triethylammonium acetate, pH 6.5, is treated with a five-fold molar excess of $AgNO_3$. After 30 min a seven-fold molar excess of dithiothreitol is added and after another 30 min the precipitated Ag^+ salt of dithiothreitol is removed by centrifugation. After reduction of the volume by rotary evaporation, the thiol-containing oligonucleotide is used immediately for further derivatization. Alternatively, the thiol-containing oligonucleotide can be stored frozen at -20 °C for months with no decomposition.

VI.4.3. Pyridyl disulfide modification of 5'-amine-containing oligonucleotides

Oligonucleotides that have been modified with an amine-terminal spacer molecule can be reacted further with the heterobifunctional cross-linking reagent SPDP (N-succinimidyl 3-(2-pyridyldithio)propionate; Pierce Chemical Company, Rockford, IL, USA). Oligonucleotides derivatized with a terminal pyridyl disulfide residue then can be coupled with sulfhydryl-containing molecules, forming a disulfide bond. Reduction of the terminal pyridyl disulfide residue releases the pyridine-2-thione leaving group and generates a terminal sulhydryl group. This procedure allows conjugation of the 5'-thiolated oligonucleotide to sulfhydryl-reactive derivatives.

First, SPDP is dissolved at a concentration of 6.2 mg/ml in DMF (makes a 20 mM stock solution). The amine-derivatized oligonucleotide is dissolved in 250 μl of 50

mM sodium phosphate, pH 7.5, and mixed with 50 µl of the SPDP solution. After reaction for 1 hour at room temperature, excess reagents are removed from the modified oligonucleotide by gel filtration.

To release the pyridine-2-thione leaving group and form the free sulfhydryl, the oligonucleotide derivative is mixed with 20 µl of 1 M dithiothreitol and incubated for 15 min at room temperature. If present in sufficient quantity, the release of pyridine-2-thione can be monitored by its characteristic absorbance at 343 nm (ε = 8.08 x 10^3 M^{-1} cm^{-1}). The thiolated oligonucleotide is purified from ecxess dithiothreitol by dialysis or gel filtration using 50 mM sodium phosphate, 1 mM EDTA, pH 7.2. The thiolated oligonucleotide is used immediately for further coupling reactions to prevent sulfhydryl oxidation.

VI.4.4. Thioester modification of 5'-amine-containing oligonucleotides

The NHS ester of SATA Pierce Chemical Company, Rockford, IL, USA) introduces a thioester moiety. The acetyl protecting group can be removed by treatment with neutral hydroxylamine. The resulting terminal sulfhydryl group can be used for subsequent conjugation to thiol-reactive molecules. The advantage of using SATA over disulfide-containing thiolation reagents such as SPDP is that the introduction of sulfhydryl residues does not include the use of a disulfide reducing agent. The pyridiyl dithiol group resulting from an SPDP thiolation must be reduced with a reducing agent such as dithiothreitol to free the sulfhydryl group. With SATA, the sulfhydryl is freed by hydroxylamine, thus eliminating the need for removal of sulfhydryl reductants prior to a conjugation reaction.

First, SATA is dissolved at a concentration of 8 mg/ml in DMF. The amine-derivatized oligonucleotide is dissolved in 250 µl of 50 mM sodium phosphate, pH 7.5, and mixed with 250 µl of the SATA solution. After reaction for 3 hours at 37 °C, excess reagents are removed from the modified oligonucleotide by gel filtration.

To deprotect the thioacetyl group, 100 µl of 50 mM hydroxylamine hydrochloride, 2.5 mM EDTA, pH 7.5, is added and reacted for 2 hours at 37 °C. The sulfhydryl-containing oligonucleotide is used immediately for further reaction with a sulfhydryl-reactive molecule.

VI.4.5. Derivatization of oligonucleotides with an aldehyde function at the 5'-terminus

The cross-linking reagent SFB (succinimidyl *p*-formylbenzoate) can be used to add aldehyde groups to amine-containing oligonucleotides. First, SFB is dissolved at a concentration of 12.35 mg/ml in acetonitrile (makes a 50 mM solution). The amine-derivatized oligonucleotide is dissolved in 250 µl of 50 mM sodium phosphate, pH 7.5, and mixed with 50 µl of the SFB solution. After reaction for 3 hours at room temperature, excess reagents are removed from the modified oligonucleotide by gel filtration.

VI.4.6. Derivatization of activated oligonucleotides with spacer molecules

To increase the flexibility and, thereby, the reactivity of oligonucleotides attached to the surface of affinity liposomes, spacer molecules are introduced between the liposomal surface and the oligonucleotides.

Method A. In method A, the heterobifunctional reagents LC-SPDP or sulfo-LC-SPDP (both Pierce Chemical Company, Rockford, IL, USA) are utilized as spacer molecules. The sulfo-NHS form of the cross-linker contains a negatively charged sulfonate group that provides water-solubility to the cross-linker.

LC-SPDP is dissolved at a concentration of 8.5 mg/ml in DMF (makes a 20 mM stock solution). If the water-soluble sulfo-LC-SPDP is used, a stock solution in water is prepared just prior to addition of an aliquot to the reaction, since an aqueous solution of the cross-linker will degrade by hydrolysis of the sulfo-NHS ester. A 10 mM stock solution of sulfo-LC-SPDP is prepared by dissolving 5.2 mg/ml water. The amine-derivatized oligonucleotide is dissolved in 250 µl of 50 mM sodium phosphate, pH 7.5, and mixed with 50 µl of the LC-SPDP solution. If the water-soluble sulfo-LC-SPDP is used, 100 µl of the sulfo-LC-SPDP solution is added. After reaction for 1 hour at room temperature, excess reagents are removed from the modified oligonucleotide by gel filtration.

Method B. In method B, water-soluble heterobifunctional derivatives of polyethylene glycol (PEG) are utilized as spacer molecules. Heterobifunctional PEG derivatives containing an amine-reactive N-hydroxysuccinimidyl (NHS) moiety and a sulfhydryl-reactive vinylsulfone (VS) moiety are especially useful, since the VS moiety is hydrolytically stable in aqueous media. At pH 7, the VS

moiety reacts selectively with sulfhydryl groups. Reaction with amino groups proceeds at higher pH, but is still relatively slow.

NHS-PEG-VS (MW 3400) or NHS-PEG-VS (MW 2000) (both Shearwater Polymers Europe, Enschede, Netherlands) is dissolved in DMF at a concentration of 10 mM. The amine-derivatized oligonucleotide is dissolved in 250 µl of 50 mM sodium phosphate, pH 7.5, and mixed with 100 µl of the NHS-PEG-VS solution. After reaction for 1 hour at room temperature, excess reagents are removed from the modified oligonucleotide by gel filtration.

VI.5. DERIVATIZATION OF LIPID MOLECULES WITH REACTIVE RESIDUES AND AFFINITY COMPONENTS

VI.5.1. Activation of phosphatidylethanolamine with a pyridyl disulfide residue

Phosphatidylethanolamine (PE) (15 mg; 20 µmol) is dissolved in 2 ml of argon-purged, anhydrous methanol containing 20 µmol of triethylamine (TEA; 2 mg) and maintained over an argon or nitrogen atmosphere. After the addition of 30 µmol LC-SPDP (Pierce Chemical Company, Rockford, IL, USA) to the PE solution, the reaction mixture is mixed well and incubated for 2 h at room temperature while maintained under an argon atmosphere. Reaction progress is determined by thin-layer chromatography (TLC) using silica gel plates developed with a mixture of chloroform : methanol:water (by volume 65:25:4). The activated PE derivative (LC-PDP-PE) develops faster on TLC than the unmodified PE. After completion of the reaction, the methanol is removed from the reaction solution by rotary evaporation and the solid is redissolved in 5 ml of chloroform. The water-soluble reaction by-products are extracted twice from the chloroform with an equal volume of 1% NaCl. The LC-PDP-PE derivative is further purified by chromatography on a column of silicic acid as described by Martin, F.J. et al. *In*: Liposomes, A Practical Approach, pp. 163-182, IRL Press, New York, 1990. Silicic acid (2 g) is dissolved in 10 ml of chloroform and poured into a syringe barrel containing a plug of glass wool at the bottom. The chloroform-dissolved lipids are applied on the silicic acid column, washed with 4 ml of chloroform, and then eluted with 4 ml each of the following series of chloroform:methanol mixtures: 4:0.25, 4:0.5, 4:0.75, and 4:1. Fractions of 2 ml are collected and monitored for the presence of purified LC-PDP-PE by TLC as described above. Finally, the chloroform is removed from the LC-PDP-PE by rotary evaporation and the derivative is stored at -20°C under a nitrogen atmosphere until use.

VI.5.2. Activation of phosphatidylethanolamine with a maleimide residue

PE (100 μmol) is dissolved in 5 ml of argon-purged, anhydrous methanol containing 100 μmol of triethylamine (TEA) and maintained over an argon or nitrogen atmosphere. After the addition of 50 mg SMPB (N-succinimidyl-4-(p-maleimidiphenyl) butyrate; Pierce Chemical Company, Rockford, IL, USA) to the PE solution, the reaction mixture is mixed well and incubated for 2 h at room temperature while maintained under an argon atmosphere. Reaction progress is determined by thin-layer chromatography (TLC) using silica gel 60-F_{254} plates (Merck, Germany) developed with a 65:25:4 (by volume) mixture of chloroform: methanol: water. The activated PE derivative develops faster on TLC than the unmodified PE. After completion of the reaction, the methanol is removed from the reaction solution by rotary evaporation and the solid is redissolved in 5 ml of chloroform. The water-soluble reaction by-products are extracted twice from the chloroform with an equal volume of 1% NaCl. The MPB-PE derivative is further purified by chromatography on a column of silicic acid as described above under VI.5.1. Fractions of 2 ml are collected and monitored for the presence of purified MPB-PE by TLC as described above. Finally, the chloroform is removed from the MPB-PE by rotary evaporation and the derivative is stored at -20°C under a nitrogen atmosphere until use.

VI.5.3. Activation of phosphatidylethanolamine with an iodoacetyl residue

PE (15 mg; 20 μmol) is dissolved in 2 ml of argon-purged, anhydrous methanol containing 20 μmol of triethylamine (TEA; 2 mg) and maintained over an argon or nitrogen atmosphere. After the addition of 30 μmol SIAXX (Pierce Chemical Company, Rockford, IL, USA) to the PE solution, the reaction mixture is mixed well and incubated for 2 h at room temperature while maintained under an argon atmosphere. Reaction progress is determined by thin-layer chromatography (TLC) using silica gel plates developed with a mixture of chloroform : methanol:water (by volume 65:25:4). The activated PE derivative (IAXX-PE) develops faster on TLC than the unmodified PE. After completion of the reaction, the methanol is removed from the reaction solution by rotary evaporation and the solid is redissolved in 5 ml of chloroform and purified by chromatography on a column of silicic acid as described above under VI.5.1. Fractions of 2 ml are

collected and monitored for the presence of purified IAXX-PE by TLC as described above. Finally, the chloroform is removed from the IAXX-PE by rotary evaporation and the derivative is stored at -20°C under a nitrogen atmosphere until use.

VI.5.4. Activation of phosphatidylethanolamine with an aldehyde group

PE (15 mg; 20 μmol) is dissolved in 2 ml of argon-purged, anhydrous methanol containing 20 μmol of triethylamine (TEA; 2 mg) and maintained over an argon or nitrogen atmosphere. After the addition of 30 μmol SFPA (succinimidyl-*p*-formylphenoxyacetate; Molecular Probes, Eugene, OR, USA) to the PE solution, the reaction mixture is mixed well and incubated for 2 h at room temperature while maintained under an argon atmosphere. Reaction progress is determined by thin-layer chromatography (TLC) using silica gel plates developed with a mixture of chloroform : methanol:water (by volume 65:25:4). The activated PE derivative (FPA-PE) develops faster on TLC than the unmodified PE. After completion of the reaction, the methanol is removed from the reaction solution by rotary evaporation and the solid is redissolved in 5 ml of chloroform and purified by chromatography on a column of silicic acid as described above under VI.5.1. Fractions of 2 ml are collected and monitored for the presence of purified FPA-PE by TLC as described above. Finally, the chloroform is removed from the FPA-PE by rotary evaporation and the derivative is stored at -20°C under a nitrogen atmosphere until use.

VI.5.5. Activation of phosphatidylethanolamine with a thioester residue

PE (15 mg; 20 μmol) is dissolved in 2 ml of argon-purged, anhydrous methanol containing 20 μmol of triethylamine (TEA; 2 mg) and maintained over an argon or nitrogen atmosphere. After the addition of 30 μmol SATP (succinimidyl acetylthiopropionate; Molecular Probes, Eugene, OR, USA) to the PE solution, the reaction mixture is mixed well and incubated for 2 h at room temperature while maintained under an argon atmosphere. Reaction progress is determined by thin-layer chromatography (TLC) using silica gel plates developed with a mixture of chloroform : methanol:water (by volume 65:25:4). The activated PE derivative (ATP-PE) develops faster on TLC than the unmodified PE. After completion of the reaction, the methanol is removed from the reaction solution by rotary evaporation and the solid is redissolved in 5 ml of chloroform. The water-soluble reaction by-products are extracted twice from the chloroform with an equal volume of 1% NaCl. The ATP-PE derivative is further purified by chromatography on a column

of silicic acid as described above under VI.5.1. Fractions of 2 ml are collected and monitored for the presence of purified ATP-PE by TLC as described above. Finally, the chloroform is removed from the ATP-PE by rotary evaporation and the derivative is stored at -20°C under a nitrogen atmosphere until use.

VI.5.6. Derivatization of lipid molecules with affinity components

VI.5.6.1. Coupling of 2,4,6-trinitrobenzene sulfonic acid to phosphatidylethanolamine

Coupling of 2,4,6-trinitrobenzene sulfonic acid (TNBSA) to PE is performed as described by van Houte, A.J. et al. (In: Liposome Technology, vol. II (G. Gregoriadis, ed.) pp. 125-139, CRC Press, Inc., Boca Raton, FL, USA, 1984). TNBSA (10 mg, 26 μmol) is dissolved in 15 ml of 10 mM sodium phosphate, pH 8.5, and mixed with 15 ml of chloroform and 30 ml of methanol containing 13 μmol PE. After vigorous stirring for 2 hrs at room temperature, 15 ml of chloroform and 15 ml of 10 mM sodium phosphate, pH 8.5, are added. Phase separation is performed by centrifugation for 10 min at 1200 x g. The chloroform layer is washed once with 20 ml of 10 mM sodium phosphate, pH 8.5, and two times with 20 ml of distilled water. To the resulting chloroform layer 60 ml of methanol are added and the TNP-PE (dissolved in a methanol: chloroform mixture of 2:1) is stored under nitrogen at -20 °C.

VI.5.6.2. Coupling of NHS-LC-biotin to phosphatidylethanolamine

Coupling of NHS-LC-biotin to to PE is performed as described by Bayer, E.A. et al. (In: Liposome Technology, vol. III (G. Gregoriadis, ed.) pp. 127-135, CRC Press, Inc., Boca Raton, FL, USA, 1984). PE (30 mg) is dissolved in 1 ml of a chloroform: methanol mixture (2: 1) containing 20 mg of NHS-LC-biotin (succinimidyl-6-(biotinamido) hexanoate; Pierce Chemical Company, Rockford, IL, USA). After the addition of 10 μl of triethylamine, the reaction mixture is incubated for 30 min at room temperature. The product (LC-biotin-PE) is purified by thin layer chromatography (TLC) on precoated silica 60 plates (Merck, Darmstadt, Germany) using a mixture of chloroform: methanol: water (80: 25: 2) for development. On the silica plates, the components are visualized with dimethylaminocinnamaldehyde, a biotin-specific reagent, or by exposure to iodine vapors. The product is scraped off the plate and extracted withchloroform: methanol (2:1). The solvent is evaporated under a stream of nitrogen, and the biotinylated lipid is stored at -20 °C.

VI.5.6.3. Coupling of 2-iminothiolane-derivatized daunorubicin to SPDP-derivatized phosphatidylethanolamine

2-Iminothiolane-derivatized daunorubicin (15 μmol) (section VI.3.1) is dissolved in 15 ml of degassed 10 mM sodium phosphate, 1 mM EDTA, pH 7.5, and mixed with 15 ml of chloroform and 15 ml of methanol containing 7 μmol SPDP-derivatized PE. After vigorous stirring for 3 hrs at room temperature, 15 ml of chloroform and 15 ml of 10 mM sodium phosphate, pH 7.5, are added. Phase separation is performed by centrifugation for 10 min at 1200 x g. The chloroform layer is washed once with 20 ml of 10 mM sodium phosphate, pH 7.5, and two times with 20 ml of distilled water. To the resulting chloroform layer 60 ml of methanol are added and the daunorubicin-PE conjugate (dissolved in a methanol: chloroform mixture of 2:1) is stored under nitrogen at -20 °C.

VI.6. PREPARATION OF REDOX MEDIATOR-CONTAINING LIPOSOMES

VI.6.1. Preparation of liposomes containing pyridyl disulfide--derivatized phosphatidylethanolamine and encapsulated *p*-aminophenol

In the follwing examples, pyridyl disulfide-derivatized dipalmitoyl phosphatidylethanolamine (PD-DPPE) is utilized as reactive lipid derivative for subsequent coupling of affinity components to the liposomal surface. Other lipid derivatives including those containing a covalently attached affinity component (e.g., biotin-derivatized dipalmitoyl phosphatidyl ethanolamine) may be used as long as they are soluble in suitable organic solvents. For encapsulation, the redox mediator *p*-aminophenol is dissolved at a concentration of 50 mM in 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer in distilled water, pH 7.4.

VI.6.1.1. Preparation of MLV liposomes using pyridyl disulfide-derivatized PE

MLV liposomes containing encapsulated *p*-aminophenol are prepared from a lipid mixture consisting, on a molar ratio basis, of dipalmitoyl phosphatidylcholine (PC), cholesterol, dipalmitoyl phosphatidylglycerol (PG), and PD-DPPE of 8:10:1:1. Using this percentage of cholesterol, the integrity of the liposomal bilayer will be stable up to a level of organic solvent addition of about 5%. This is important for subsequent coupling of affinity components added to the liposome suspension as a concentrated stock dilution in an organic solvent.

The lipid mixture solved in organic solvent (approximatey a total of 60 μmol/ml preparation) is pipetted into a round-bottom flask and then dried under reduced pressure at or close to its transition temperature (the highest transition temperature of any one lipid in the mixture is taken into consideration). Thereafter, the lipid mixture is hydrated with *p*-aminophenol dissolved at a concentration of 50 mM in 10 mM HEPES buffer, pH 7.4, by vortexing for at least 10 min in a water bath at or above the transition temperature of the lipid mixture. Next, the lipid mixture is mechanically shaken in the water bath for about 30 min and after another 30 min at room temperature (without skaking), the liposome mixture is filtered through 0.4 μm nucleopore filter under nitrogen pressure. Sometimes it is necessary, to perform sequential filtering starting from 1.0, 0.6., and then 0.4 μm. The MLVs are dialyzed against 10 mM phosphate-buffered saline, pH 7.4 (at least 1: 100 volume; three or four changes; 1 hr each; then overnight at 4 °C). To obtain a more concentrated liposome suspension, it can be centrifuged at 100,000 x g (80 min at 20 °C).

VI.6.1.2. Preparation of SUV liposomes using pyridyl disulfide-derivatized PE

SUVs are made from the MLV. After the first step in the MLV procedure (hydration by vortexing), the lipid mixture is transferred to a sonicating flask, equipped with a long neck and cap with in and out spouts for nitrogen. Nitrogen is flushed through the flask, then a light stream of nitrogen is left going into the tube and the outlet is closed. The flask is placed into a sonicator (e.g., Laboratory Supplies Company, Hicksville, NY, USA) at or above the highest transition temperature of any one lipid in the mixture. Sonication is performed for 30 to 60 min until the mixture appears opalescent. Thereafter, the SUVs are allowed to equilibrate for approximately 30 min at room temperature before uncaptured redox mediator is removed by dialysis against 10 mM phosphate-buffered saline, pH 7.4 (at least 1: 100 volume; three or four changes; 1 hr each; then overnight at 4 °C). To obtain a more concentrated liposome suspension, it can be centrifuged at 100,000 x g (80 min at 20 °C).

VI.6.1.3. Preparation of LUV liposomes using pyridyl disulfide-derivatized PE

For the preparation of LUV liposomes according to the reverse-phase evaporation technique of Szoka, F.C. et al. (Proc. Natl. Acad. Sci. USA 75, 4194, 1978), a lipid formulation consisting, on a molar ratio basis, of PC, cholesterol, PG, and PD-DPPE of 8:10:1:1 is used. After mixing of the lipids solved in organic solvent, the solvent is evaporated to dryness on a rotary evaporator. The dried lipids are

redissolved in isopropyl ether, freshly redistilled from sodium bisulfite, to a concentration of approximately 20 μmol/ml ether and transferred to a 50 ml screw-cap Erlenmeyer flask. Thereafter, the aqueous phase consisting of 50 mM *p*-aminophenol in 10 mM HEPES buffer in distilled water, pH 7.4, is added directly to the lipid solution in a ratio of 1:3 with ether. The flask is sealed with nitrogen, contents are mixed very well, and the mixture is sonicated at room temperature for at least 5 min until the mixture looks homogeneous. The organic phase is then removed by rotary evaporation under reduced pressure initially at about 450 mm Hg for small preparations and 550 mm Hg for larger preparations. When gel forms, vacuum is increased gradually to a maximum of 700 to 750 mm Hg. Foaming during this process can be eliminiated by quick flushing of nitrogen into the flask. The temperature is also increased gradually from room temperature to about 37 °C towards the end of evaporation. At the end of the process, the residue is slightly less in volume than the original aqueous phae (at this stage, no odor of isopropyl ether should be detectable). Thereafter, the SUVs are allowed to equilibrate for approximately 30 to 60 min at room temperature, then extruded through 0.4 _m nucleopore filter under nitrogen pressure. Sometimes it is necessary, to perform sequential filtering starting from 1.0, 0.6., and then 0.4 _m. Uncaptured redox mediator is removed by dialysis against 10 mM phosphate-buffered saline, pH 7.4 (at least 1: 100 volume; three or four changes; 1 hr each; then overnight at 4 °C). To obtain a more concentrated liposome suspension, it can be centrifuged at 100,000 x g (80 min at 20 °C).

VI.6.2. Preparation of temperature-sensitive liposomes containing phosphatidylglycerol and encapsulated *p*-aminophenol

Since the goal is to produce affinity liposomes that undergo a sharp increase in release of encapsulated redox mediators at T_m, it is necessary to minimize lipid-soluble contaminating substances. This condition is usually met by using synthetic lipids of greater than 99% fatty acid purity. In the described examples, the synthetic lipids DPPC (T_m = 41 °C), DPPG (T_m = 41 °C), and DSPC (T_m = 54 °C) are utilized. Incorporation of DPPG into the liposomal bilayer provides periodate-oxidizable vicinal hydroxyl groups which can be utilized to generate surface-attached aldehyde functions for covalent coupling of affinity components. For encapsulation, the redox mediator *p*-aminophenol is dissolved at a concentration of 50 mM in 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer in distilled water, pH 7.4.

VI.6.2.1. Preparation of temperature-sensitive SUV liposomes

For the preparation of temperature-sensitive SUV-affinity liposomes, the lipid formulation consists of DPPC, DPPG, and DSPC at a molar ratio of 6.5: 0.5: 3.0. The DSPC is added to counterbalance the lowering of T_m due to the small radius of curvature of SUV. The lipids are dried from benzene onto a glass vial under a stream of argon and lyophilized overnight. A 5 ml aliquot of the 50 mM *p*-aminophenol solution in 10 mM HEPES, pH 7.4, is warmed in a water bath to 50 °C and added to the vial containing approximately a total of 40 mg lipids, also at 50 °C. At the same temperature, the suspension is hydrated with repeated votex-mixing for about 15 min. Thereafter, the suspension is sonicated under argon to form SUV at 50 °C. After clarification of the suspension (usually within 5 min of sonication), the suspension is maintained above T_m for 15 min and centrifuged briefly at low speed to remove insoluble material. Finally, non-encapsulated *p*-aminophenol is removed by size exclusion chromatography on Sepharose 4B (Pharmacia) equilibrated in 20 mM sodium phosphate, 0.15 M NaCl, pH 7.4.

VI.6.2.2. Preparation of temperature-sensitive LUV liposomes

For the preparation of temperature-sensitive LUV-affinity liposomes according to the reverse-phase evaporation technique of Szoka, F.C. et al. (Proc. Natl. Acad. Sci. USA 75, 4194, 1978), a lipid formulation consisting of DPPC and DPPG at a molar ratio of 9: 1 is used. After mixing of the lipids solved in organic solvent, the solvent is evaporated to dryness on a rotary evaporator. The dried lipids (approximately a total of 125 mg) are redissolved in an organic phase consisting of 4 ml of chloroform and 8 ml of isopropyl ether, freshly washed with 10% sodium bisulfite. The mixture is transferred to a 50 ml screw-cap Erlenmeyer flask. The aqueous phase consists of 10 mM HEPES buffer in distilled water, pH 7.4, containing 50 mM *p*-aminophenol. The organic phase (12 ml) and the aqueous phase (4 ml) are warmed to 50 °C and combined. The screw-cap Erlenmeyer flask is then filled with nitrogen gas and sealed with Teflon tape. The organic / aqueous mixture is placed in a cylindrical bath-type sonicator (e.g., Laboratory Supplies Company, Hicksville, N.Y., USA) filled with water at 45 to 50 °C, and sonicated for 5 min to form a milky, white, homogeneous emulsion. The emulsion is then transferred to a 125 ml tear-drop-shaped rotary evaporation flask. The water around the flask is kept at 50 °C. Upon lowering the pressure, the organic phase is drawn off. During this process the emulsion foams exuberantly and requires careful venting to adjust the pressure. After a period of 10 to 20 min, it is useful to add approximately 2 ml of additional HEPES buffer to replace the

amount that is lost during the evaporation process. The procedure is finished when no foaming occurs at a pressure of approximately 150 mm Hg. The newly formed liposomes are allowed to anneal at 50 °C in a water bath for 30 min or longer. LUV affinity liposomes of well-defined size are formed by extrusion of this suspension through polycarbonate membranes above T_m. The liposomes are then rapidly cooled to room temperature in an ice bath and dialyzed over 24 hrs against two 1000 ml volumes of HEPES buffer to remove non-encapsulated *p*-aminophenol.

VI.6.2.3. Preparation of temperature-sensitive MLV liposomes

For the preparation of temperature-sensitive MLV-affinity liposomes, a lipid formulation consisting of DPPC and DPPG at a molar ratio of 9: 1 is used. After mixing of the lipids (aprproximately a total of 125 mg) solved in organic solvent, the solvent is evaporated to dryness on the wall of a 100 ml round-bottom fask. The aqueous phase consists of 10 mM HEPES buffer in distilled water, pH 7.4, containing 50 mM *p*-aminophenol. After heating of the aqueous phase to 50 °C, 4 ml are transferred to the flask containing the dried lipids, taking care to keep the flask above the T_m of the mixture. The flask is filled with nitrogen gas, closed with a glass stopper, and sealed with Teflon tape. Thereafter, the lipids are hydrated by repeated cycles of vortex-mixing for 15 sec followed by 1.5 min of incubation in a 50 °C water bath. The suspension is cycled 20 times in this manner and then allowed to anneal 30 min or longer in the 50 °C water bath. The liposomes are then rapidly cooled to room temperature in an ice bath and dialyzed over 24 hrs against two 1000 ml volumes of HEPES buffer to remove non-encapsulated *p*-aminophenol.

VI.7. DERIVATIZATION OF INTACT LIPOSOMES WITH REACTIVE RESIDUES

VIII.7.1. Derivatization of intact liposomes with pyridyl disulfide residues

First, LUV, SUV, and MLV liposomes containing encapsulated redox mediators are prepared from a lipid mixture consisting, on a molar ratio basis, of PC, cholesterol, PG, and PE of 8:10:1:1 as described in section VI.6.1. Other lipid recipes may be used as long as they contain about this percentage of PE. In addition, if this level of cholesterol is maintained in the liposome, then the integrity of the bilayer will be stable up to a level of organic solvent addition of about 5%. This factor is important for adding an aliquot of the cross-linker to the liposome

suspension as a concentrated stock dilution in an organic solvent. Any method of liposome formation may be used.

Derivatization of PE-liposomes with pyridyl disulfide residues can be performed with SPDP, LC-SPDP, or sulfo-LC-SPDP (all of Pierce Chemical Company, Rockford, IL, USA). SPDP is dissolved at a concentration of 6.2 mg/ml in DMF (makes a 20 mM stock solution). Alternatively, LC-SPDP is dissolved at a concentration of 8.5 mg/ml in DMF (also makes a 20 mM stock solution). If the water-soluble sulfo-LC-SPDP is used, a stock solution in water is prepared just prior to addition of an aliquot to the reaction, since an aqueous solution of the cross-linker will degrade by hydrolysis of the sulfo-NHS ester. The sulfo-NHS form of the cross-linker contains a negatively charged sulfonate group that prevents the reagent from penetrating lipid bilayers. Thus, only the outer surfaces of the liposomes are activated using sulfo-LC-SPDP. A 10 mM stock solution of sulfo-LC-SPDP is prepared by dissolving 5.2 mg/ml water.

To each milliliter of the liposome suspension to be modified, 25 - 50 μl of the stock solution of either SPDP or LC-SPDP in DMF is added. If sulfo-LC-SPDP is used, 50 - 100 μl of the stock solution in water is added to each milliliter of the liposome suspension. The reaction mixture is vortexed and reacted for 30 min at room temperature. Longer reaction times, even overnight, have no adverse effects. Finally, the modified liposomes are purified from reaction by-products by dialysis or gel filtration using Sephadex G-50. The derivatized liposomes may be used immediately for subsequent coupling reactions or stored in a lyophilized state in the presence of sorbitol as described by Friede, M. et al., Anal. Biochem. 211, 117, 1993.

VI.7.2. Derivatization of intact liposomes with aldehyde functions

First, LUV, SUV, and MLV liposomes containing encapsulated redox mediators are prepared from a lipid mixture consisting, on a molar ratio basis, of PC, cholesterol, PG, and other glycolipids of 8:10:1:1 as described in section VI.6.1. The other glycolipids that can be incorporated include phosphatidyl inositol, lactosylceramide, galactose cerebroside, or various gangliosides. Other liposome compositions may be used (e.g., recipes without cholesterol), as long as a periodate-oxidizable component containing vicinal hydroxyls is present. Any method of liposome formation may be used.

Temperature-sensitive SUV liposomes containing phosphatidylglycerol and encapsulated redox mediators are prepared from a lipid mixture consisting, on a molar ratio basis, of DPPC, DPPG, and DSPC at a molar ratio of 6.5: 0.5: 3.0 as described in section VI.6.2.1. Temperature-sensitive LUV and MLV liposomes containing phosphatidylglycerol and encapsulated redox mediators are prepared from a lipid mixture consisting, on a molar ratio basis, of DPPC and DPPG at a molar ratio of 9: 1 as described in sections VI.6.2.2 and VI.6.2.3, respectively.

Sodium periodate is dissolved in water to a concentration of 0.6 M (128 mg of sodium periodate/ ml of H_2O) and 200 µl of this stock solution is added with stirring to each milliliter of PG-liposomes (5 mg/ml) suspended in 20 mM sodium phosphate, 0.15 M NaCl, pH 7.4. After incubation at room temperature for 30 min in the dark, the oxidized liposomes are dialyzed against 20 mM sodium borate, o.15 M NaCl, pH 8.4, to remove unreacted periodate. This buffer is optimal for subsequent coupling with amine-containing affinity components such as antibodies. Alternatively, unreacted periodate can be removed by gel filtration using a column of Sephadex G-50. The periodate-oxidized liposomes may be used immediately for subsequent coupling reactions or stored in a lyophilized state in the presence of sorbitol as described by Friede, M. et al., Anal. Biochem. 211, 117, 1993.

VI.8. COVALENT ATTACHMENT OF AFFINITY COMPONENTS TO INTACT REDOX MEDIATOR-CONTAINING LIPOSOMES

VI.8.1. Covalent attachment of intercalating agents to intact liposomes

VI.8.1.1. Coupling of vinylsulfone-derivatized daunorubicin to intact thioester-derivatized liposomes

A suspension (5 mg/ml) of acetylthiopropionate-derivatized liposomes (sections VI.5.5. and VI.6.) in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.5, is mixed with 0.5 M hydroxylamine hydrochloride in 50 mM sodium phosphate, 25 mM EDTA, pH 7.5 (100 µl per ml of liposome suspension). After 2 hrs at room temperature, the deacetylated liposomes are purified by gel filtration on Sephadex G-50 (Pharmacia) equlibrated with 50 mM sodium phosphate, 150 mM NaCl, 2.5 mM EDTA, pH 7.5, and used immediately to couple vinylsulfone-derivatized daunorubicin.

Daunorubicin derivatized at its daunosamine moiety with the heterobifunctional poly (ethylene glycol) (PEG) derivative NHS-PEG-VS (section VI.3.4.) is dissolved

in DMF at a concentration of 10 mM. From this solution, 25 to 50 µl are added to each ml of purified sulfhydryl-containing liposomes. The bilayer of liposomes containing 50% cholesterol will be stable up to a level of organic solvent addition of about 5%. After 3 hrs at room temperature, the daunorubicin-derivatized liposomes are purified by gel filtration on Sepharose 4B (Pharmacia) equlibrated with 20 mM sodium phosphate, 150 mM NaCl, pH 7.4.

VI.8.1.2. Coupling of sulfhydryl-derivatized daunorubicin to intact LC-SPDP-derivatized liposomes

Daunorubicin derivatized at its daunosamine moiety with 2-iminothiolane (section VI.3.1.) is dissolved in DMF at a concentration of 10 mM. From this solution, 25 to 50 µl are added to each ml of LC-SPDP-derivatized liposomes (sections VI.5.1. and VI.6.) suspended in 0.1 M sodium phosphate, 0.15 M NaCl, 2.5 mM EDTA, pH 7.5 (5mg lipid/ml). The bilayer of liposomes containing 50% cholesterol will be stable up to a level of organic solvent addition of about 5%. After 3 hrs at room temperature, the daunorubicin-derivatized liposomes are purified by gel filtration on Sepharose 4B (Pharmacia) equlibrated with 20 mM sodium phosphate, 150 mM NaCl, pH 7.4.

VI.8.2. Covalent attachment of oligonucleotides to intact liposomes

VIII.8.2.1. Coupling of vinylsulfone-derivatized oligonucleotides to intact thioester-derivatized liposomes

Acetylthiopropionate-derivatized liposomes (sections VI.5.5. and VI.6.) suspended in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.5, at a concentration of 5 mg lipid/ml, are mixed with 0.5 M hydroxylamine hydrochloride in 50 mM sodium phosphate, 25 mM EDTA, pH 7.5 (100 µl per ml of liposome suspension). After 2 hrs at room temperature, the deacetylated liposomes are purified by gel filtration on Sephadex G-50 (Pharmacia) equlibrated with 50 mM sodium phosphate, 150 mM NaCl, 2.5 mM EDTA, pH 7.5, and used immediately to couple vinylsulfone (VS)-derivatized oligonucleotides prepared from 5'-amine-containing oligonucleotides (section VI.4.1.) by reaction with the heterobifunctional PEG derivative NHS-PEG-VS (compare section VI.4.6.). An aliquot of 500 µl of 50 mM sodium phosphate, pH 7.5, containing the oligonucleotide-PEG-VS derivative at a concentration of 1 mM, is added to each ml of sulfhydryl-derivatized liposomes. After 3 hrs at room temperature, the oligonucleotide-derivatized liposomes are purified by gel filtration on Sepharose 4B (Pharmacia) equlibrated with 20 mM sodium phosphate, 150 mM NaCl, pH 7.4.

VI.8.2.2. Coupling of sulfhydryl-derivatized oligonucleotides to intact LC-SPDP-derivatized liposomes

An aliquot of 500 µl of 10 mM sodium phosphate, 150 mM NaCl, 10 mM EDTA, pH 7.2, containing 5'-sulfhydryl derivatives of oligonucleotides (section VI.4.2.) at a concentration of 1 mM, is added to each ml of LC-SPDP-derivatized liposomes (sections VI.5.1. and VI.6.) suspended in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.5 (5mg lipid/ml). After 3 hrs at room temperature, the oligonucleotide-derivatized liposomes are purified by gel filtration on Sepharose 4B (Pharmacia) equlibrated with 20 mM sodium phosphate, 150 mM NaCl, pH 7.5.

VI.8.2.3. Coupling of hydrazide-derivatized oligonucleotides to intact aldehyde-derivatized liposomes

An aliquot of 500 µl of 10 mM sodium phosphate, 150 mM NaCl, 10 mM EDTA, pH 7.2, containing oligonucleotide-5'-adipic acid hydrazide derivatives (section VI.4.1.) at a concentration of 1 mM, is added to each ml of aldehyde-derivatized liposomes (section VI.7.2.) suspended in 20 mM sodium borate, 0.15 M NaCl, pH 8.4 (5mg lipid/ml). After 3 hrs stirring at room temperature, the oligonucleotide-derivatized liposomes are purified by gel filtration on Sepharose 4B (Pharmacia) equlibrated with 20 mM sodium phosphate, 150 mM NaCl, pH 7.5.

For further stabilization of the formed hydrazone bonds, the oligonucleotide-derivatized liposomes may be treated with a reducing agent such as cyanoborohydride prior to purification by gel filtration. In a fume hood, 125 mg of sodium cyanoborohydride is dissolved in 1 ml water (makes a 2 M solution). This solution is allowed to sit for 30 min to eliminate most of the hydrogen-bubble evolution that could effect the liposome suspension. An aliquot of 10 µl of the cyanoborohydride solution is added to each ml of oligonucleotide-derivatized liposomes. After reaction at 4 °C overnight, the oligonucleotide-derivatized liposomes are purified by gel filtration on Sepharose 4B (Pharmacia) equlibrated with 20 mM sodium phosphate, 150 mM NaCl, pH 7.5.

VI.8.3. Covalent attachment of proteinaceous affinity components to intact liposomes

Proteinaceous affinity components include antibodies (e.g., with specificity for haptens and double- and/or triple-stranded nucleic acids), enzymes (e.g., dihydrofolate reductase), and streptavidin (or avidin). Coupling of these

proteinaceous affinity components to liposomes occasionally may include liposome aggregation. This may be due to the unique properties or concentration of the protein used, or it may be a result of liposome-to-liposome cross-linking during the conjugation process. Adjusting the amount of proteinaceous affinity component in the reaction mixture as well as the relative amount of reactive residues (e.g., pyridyl disulfide residues) attached to each proteinaceous affinity component may have to be done to solve an aggregation problem.

VI.8.3.1. Coupling of sulfhydryl-derivatized proteinaceous affinity components to intact LC-SPDP-derivatized liposomes

a) *Coupling of sulfhydryl-derivatized antibodies to intact LC-SPDP-derivatized liposomes.* To 1.0 ml of 20 mM sodium phosphate, 150 mM NaCl, pH 7.4, containing 10 mg of IgG antibody, 16 µl of 20 mM LC-SPDP (Pierce Chemical Company, Rockford, IL, USA) in DMF (8.5 mg/ml) is added and mixed. This gives a molar ratio of 5 mol LC-SPDP per mol of IgG. After incubation for 30 min at room temperature, the solution is subjected to gel filtration on Sephadex G-25 (Pharmacia) equilibrated with 0.1 M sodium acetate, 0.15 M NaCl, pH 4.5. At this point, the derivative is stable and may be stored. The degree of substitution can be determined with an aliquot of the purified LC-SPDP-derivatized antibody at a known protein concentration by measurement of the OD_{343} (resulting from the release of pyridine-2-thione; extinction coefficient of 8,080 $M^{-1}cm^{-1}$) after the addition of dithiothreitol (DTT) to a final concentration of 50 mM.

For reduction of the pyridyl disulfide residues, to the purified LC-SPDP-derivatized antibody in 0.1 M sodium acetate, 0.15 M NaCl, pH 4.5, 0.5 M DTT is added to a final concentration of 50 mM. At pH 4.5, DTT reduces the pyridyl disulfide bonds, but not the intrinsic aliphatic disulfide bonds of antibodies. After 20 min at room temperature, the antibodies are separated from DTT by a second column passage on Sephadex G-25 (Pharmacia) equilibrated with 50 mM sodium phosphate, 150 mM NaCl, 10 mM EDTA, pH 7.4.

The purified sulfhydryl-derivatized antibodies are used immediately for coupling to LC-SPDP-derivatized liposomes (sections VIII.5.1. and VIII.6.) suspended at a concentration of 5 mg lipid/ml in 50 mM sodium phosphate, 150 mM NaCl, 10 mM EDTA, pH 7.4. After the addition of an equal volume of sulfhydryl-derivatized antibodies (adjusted to 5 mg protein/ml) in 50 mM sodium phosphate, 150 mM NaCl, 10 mM EDTA, pH 7.4, to the liposome suspension, the reaction mixture is

stirred overnight at room temperature in a nitrogen or argon atmosphere to prevent lipid oxidation. Non-coupled antibodies are removed by gel filtration on Sepharose 4B (Pharmacia) equlibrated with 50 mM sodium phosphate, 150 mM NaCl, pH 7.4.

b) *Coupling of sulfhydryl-derivatized streptavidin to intact LC-SPDP-derivatized liposomes.*_To each ml of 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2, containing streptavidin at a concentration of 10 mg/ml, 25 µl of SATA (N-succinimidyl S-acetylthioacetate; Pierce Chemical Company, Rockford, IL, USA) dissolved in DMSO at a concentration of 13 mg/ml are added (for different concentrations of streptavidin in the reaction medium, the amount of SATA is adjusted proportionally, but not beyond 10% DMSO in the aqueous reaction medium). After 30 min at room temperature, the SATA-derivatized streptavidin is purified by gel filtration on Sephadex G-25 equilibrated with 0.1 M sodium phosphate, 0.15 M NaCl, 10 mM EDTA, pH 7.2. At this point, the derivative is stable and may be stored.

For deprotection of the acetylated sulfhydryl groups, each ml of SATA-derivatized streptavidin is mixed with 100 µl of 0.5 M hydroxylamine in 0.1 M sodium phosphate, 10 mM EDTA, pH 7.2. After 2 hrs at room temperature, the thiolated streptavidin is purified by gel filtration on Sephadex G-25 equilibrated with 0.05 M sodium phosphate, 0.15 M NaCl, 10 mM EDTA, pH 7.4.

Purified sulfhydryl-derivatized streptavidin is used immediately for coupling to LC-SPDP-derivatized liposomes (sections VI.5.1. and VI.6.) suspended at a concentration of 5 mg lipid/ml in 50 mM sodium phosphate, 150 mM NaCl, 10 mM EDTA, pH 7.4. After the addition of an equal volume of sulfhydryl-derivatized streptavidin (adjusted to 5 mg protein/ml) in 50 mM sodium phosphate, 150 mM NaCl, 10 mM EDTA, pH 7.4, to the liposome suspension, the reaction mixture is stirred overnight at room temperature in a nitrogen or argon atmosphere to prevent lipid oxidation. Non-coupled streptavidin is removed by gel filtration on Sepharose 4B (Pharmacia) equlibrated with 50 mM sodium phosphate, 150 mM NaCl, pH 7.4.

c) *Coupling of sulfhydryl-derivatized dihydrofolate reductase (DHFR) to intact LC-SPDP-derivatized liposomes.* To each ml of 50 mM sodium phosphate, 0.15 M NaCl, pH 7.2, containing dihydrofolate reductase (from chicken liver; Sigma-

Aldrich, Deisenhofen, Germany) at a concentration of 1 mg/ml, a 10-fold molar excess of sulfo-LC-SPDP (Pierce Chemical Company, Rockford, IL, USA) (freshly dissolved in 50 mM sodium phosphate, 0.15 M NaCl, pH 7.2, at a concentration of 10 mM) is added. After 30 min at room temperature, the LC-SPDP-derivatized DHFR is purified by gel filtration on Sephadex G-25 equilibrated with 0.1 M sodium phosphate, 0.15 M NaCl, 10 mM EDTA, pH 7.2. At this point, the derivative is stable and may be stored. The degree of substitution can be determined with an aliquot of the purified LC-SPDP-derivatized DHFR at a known protein concentration by measurement of the OD_{343} (resulting from the release of pyridine-2-thione; extinction coefficient of 8,080 $M^{-1}cm^{-1}$) after the addition of dithiothreitol (DTT) to a final concentration of 50 mM.

For reduction of the pyridyl disulfide residues, to purified LC-SPDP-derivatized DHFR in 50 mM sodium phosphate, 0.15 M NaCl, pH 7.4, 0.5 M DTT is added to a final concentration of 50 mM. Since DHFR contains no disulfide bonds, the incorporated pyridyl disulfide residues can be deprotected with DTT without detrimental effects to the enzyme. After 20 min at room temperature, the sulfhydryl-derivatized DHFR is separated from DTT by a second column passage on Sephadex G-25 (Pharmacia) equilibrated with 50 mM sodium phosphate, 150 mM NaCl, 10 mM EDTA, pH 7.4.

Purified sulfhydryl-derivatized DHFR is used immediately for coupling to LC-SPDP-derivatized liposomes (sections VI.5.1. and VI.6.) suspended at a concentration of 5 mg lipid/ml in 50 mM sodium phosphate, 150 mM NaCl, 10 mM EDTA, pH 7.4. After addition of 2.5 ml of sulfhydryl-derivatized DHFR (adjusted to approximately 0.6 mg protein/ml) in 50 mM sodium phosphate, 150 mM NaCl, 10 mM EDTA, pH 7.4, to each ml of the liposome suspension, the reaction mixture is stirred overnight at room temperature in a nitrogen or argon atmosphere to prevent lipid oxidation. Non-coupled DHFR is removed by gel filtration on Sepharose 4B (Pharmacia) equlibrated with 50 mM sodium phosphate, 150 mM NaCl, pH 7.4.

VI.8.3.2. Coupling of sulfhydryl-derivatized proteinaceous affinity components to intact SMPB-derivatized liposomes

Derivatization of proteinaceous affinity components with sulfhydryl groups is performed as described in VI.8.3.1. In this example, sulfhydryl-derivatized IgG antibodies are coupled to intact SMPB-derivatized liposomes.

After purification by gel filtration (VI.8.3.1.) sulfhydryl-derivatized IgG antibodies are used immediately for coupling to SMPB-derivatized liposomes (sections VI.5.2. and VI.6.) suspended at a concentration of 5 mg lipid/ml in 50 mM sodium phosphate, 150 mM NaCl, 10 mM EDTA, pH 7.4. After the addition of an equal volume of sulfhydryl-derivatized antibodies (adjusted to 5 mg protein/ml) in 50 mM sodium phosphate, 150 mM NaCl, 10 mM EDTA, pH 7.4, to the liposome suspension, the reaction mixture is stirred overnight at room temperature in a nitrogen or argon atmosphere to prevent lipid oxidation. Non-coupled antibodies are removed by gel filtration on Sepharose 4B (Pharmacia) equlibrated with 50 mM sodium phosphate, 150 mM NaCl, pH 7.4.

VI.8.3.3. Coupling of non-derivatized proteinaceous affinity components to intact aldehyde-derivatized liposomes

In this example, non-derivatized IgG antibodies are coupled to periodate-oxidized liposomes (Method A) or to glutaraldehyde-activated liposomes (Method B).

Method A. To each ml of periodate-oxidized liposomes (section VI.7.2.) suspended in 20 mM sodium borate, 0.15 M NaCl, pH 8.4, at a concentration of 5 mg lipid/ml, 500 µl of 20 mM sodium borate, 0.15 M NaCl, pH 8.4, containing non-derivatized IgG antibodies at a concentration of 10 mg/ml, are added. After 2 hrs stirring at room temperature, the Schiff base interactions between the aldehydes on the liposomes and the amines on the antibodies are stabilized by reduction with cyanoborohydride. In a fume hood, 125 mg of sodium cyanoborohydride is dissolved in 1 ml water (makes a 2 M solution). This solution is allowed to sit for 30 min to eliminate most of the hydrogen-bubble evolution that could effect the liposome suspension. An aliquot of 10 µl of the cyanoborohydride solution is added to each ml of antibody-derivatized liposomes. After reaction at 4 °C overnight, the antibody-derivatized liposomes are purified by gel filtration on Sepharose 4B (Pharmacia) equlibrated with 20 mM sodium phosphate, 150 mM NaCl, pH 7.5.

Method B. Phosphatidyl ethanolamine (PE) liposomes containing encapsulated redox mediators are prepared as described in section VI.7.1. and suspended in 0.1 M sodium phosphate, 0.15 M NaCl, pH 6.8, at a concentration of 5 mg lipid/ml. All buffers are degassed and bubbled with nitrogen or argon prior to use. After the addition of glutaraldehyde to a final concentration of 1.25%, the liposome suspension is reacted overnight at room temperature under a nitrogen

atmosphere, and then purified from exess glutaraldehyde by gel filtration on Sephadex G-50 (Pharmacia) equilibrated with 0.1 M sodium phosphate, 0.15 M NaCl, pH 6.8.

To each ml of purified glutaraldehyde-activated liposomes, 1 ml of 0.5 M sodium carbonate, pH 9.5, containing non-derivatized antibodies at a concentration of 10 mg/ml, is added (ratio of 2 mg antibody per mg lipid). After 3 hrs stirring at room temperature under a nitrogen atmosphere, any excess aldehydes and the Schiff base interactions between the aldehydes on the liposomes and the amines on the antibodies are reduced with cyanoborohydride. In a fume hood, 125 mg of sodium cyanoborohydride is dissolved in 1 ml water (makes a 2 M solution). This solution is allowed to sit for 30 min to eliminate most of the hydrogen-bubble evolution that could effect the liposome suspension. An aliquot of 10 µl of the cyanoborohydride solution is added to each ml of antibody-derivatized liposomes. After reaction at 4 °C overnight, the antibody-derivatized liposomes are purified by gel filtration on Sepharose 4B (Pharmacia) equlibrated with 20 mM sodium phosphate, 150 mM NaCl, pH 7.5.

VI.8.3.4. Coupling of hydrazide-derivatized proteinaceous affinity components to intact aldehyde-derivatized liposomes

In this example, streptavidin is derivatized with adipic acid dihydrazide according to a method of Bayer, E.A. et al. (Anal. Biochem. 161, 123, 1987). Adipic acid dihydrazide (160 mg) dissolved in 5 ml of 0.1 M sodium phosphate, pH 6 (some heating may be required during solubilization of the compound) is mixed with 50 mg streptavidin. To this solution, 160 mg of water-soluble 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride is added and mixed thoroughly. After reaction for 4 hrs at room temperature, hydrazide-derivatized streptavidin is purified by gel filtration on Sephadex G-50 (Pharmacia) equilibrated with 0.1 M sodium phosphate, 0.15 M NaCl, pH 6.8. Hydrazide-derivatized streptavidin may be stored as a freeze-dried preparation without loss of ativity.

Coupling of hydrazide-derivatized streptavidin to aldehyde-derivatized liposomes is performed as described in section VI.8.3.3. Stabilization by reduction with cyanoborohydride of the hydrazone linkages formed between the aldehydes on the liposomes and the hydrazide residues on streptavidin may be omitted, since hydrazone linkages provide a much higher stability than Schiff base interactions. However, the addition of a reductant during hydrazide/aldehyde reactions increases the efficiency and yield of the reaction.

VI.8.3.5. Incorporation of proteinaceous affinity component-palmitate conjugates into liposomal membranes

In this example, non-derivatized antibodies are coupled to palmitic acid derivatized with an N-hydroxysuccinimide ester, followed by incorporation of the antibody-palmitate conjugate into intact liposomes by detergent dialysis according to Huang, A. et al. (J. Biol. Chem. 255, 8015, 1980).

To 30 ml of dry ethyl acetate containing 3.45 mg of (NHS) 30 mmol of palmitic acid is added. The solution is kept under a nitrogen atmosphere. After further addition of 6.18 g of dicyclohexyl carbodiimide dissolved in 10 ml of dry ethyl acetate, the solution is reacted overnight at room temperature while maintaining the nitrogen atmosphere. Insoluble dicyclohexyl urea is removed by filtration using a glass fiber filter pad and vacuum, and the solvent is removed from the filtered solution by rotary evaporation under vacuum. For purification of the NHS-palmitate by recrystallization, the activated fatty acid is dissolved in a minimum quantity of hot ethanol and immediately filtered through a filter funnel containing a fluted glass fiber filter pad, both of which have been warmed to the same temperature as the ethanol solution. The NHS-palmitate is recrystallized overnight at room temperature, the solvent is removed by filtration, and the solid is dried under vacuum in a desiccator. The purity of NHS-palmitate can be analyzed by TLC on silica plates using a mixture of chloroform: petroleum diethyl ether (bp 40-60 °C) of 8: 2 as solvent. NHS and NHS-palmitate may be detected by staining with 10% hydroxylamine in 0.1 M NaOH followed after 2 min by a 5% solution of $FeCl_3$ in 1.2 M HCl (creates red-coloured spots).

For conjugation of NHS-palmitate to antibodies, 44 μg purified NHS-palmitate dissolved in 20 mM sodium phosphate, 0.15 M NaCl, 2% deoxycholate, pH 7.4, are mixed with 2 mg antibody and incubated for 10 hrs at 37 °C. Excess palmitic acid is removed by gel filtration on Sephadex G-75 (Pharmacia) equilibrated with 20 mM sodium phosphate, 0.15 M NaCl, 0.15% deoxycholate, pH 7.4.

For incorporation of palmitate-antibody conjugates into intact liposomes, palmitate-antibody conjugates in 20 mM sodium phosphate, 0.15 M NaCl, 0.15% deoxycholate, pH 7.4, are added in a ratio of 20: 1 (w/w) to intact liposomes in 20 mM sodium phosphate, 0.15 M NaCl, pH 7.4. After the addition of deoxycholate to a final concentration of 0.7%, the suspension is mixed thoroughly using a vortex

mixer, and the liposomes are dialyzed against 20 mM sodium phosphate, 0.15 M NaCl, pH 7.4.

VI.8.4. Covalent attachment of proteinaceous affinity components derivatized with low molecular affinity components to intact liposomes

For the preparation of preformed complexes of affinity liposomes capable of binding to amplification polymers, liposomes may be prepared containing surface-attached proteinaceous affinity components (e.g., antibodies) which are derivatized with low molecular weight affitnity components (e.g., biotin). The proteinaceous affinity component such as an anti-hapten antibody can mediate binding of the liposomes to hapten-derivatized amplification polymers and the biotin residues complexation of the liposomes via streptavidin as bridging molecule. Covalent attachment of proteinaceous affinity components derivatized with low molecular affinity components to intact liposomes is performed as described in section VI.8.3.

VI.8.4.1. Derivatization of antibodies with biotin residues

NHS-LC-biotin (succinimidyl-6-(biotinamido) hexanoate; Pierce Chemical Company, Rockford, IL, USA) is dissolved in DMF at a concentration of 40 mg/ml. An aliquot of 50 µl of this solution is added in two aliquots (apportioned 10 min apart) to each ml of 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2, containing the IgG antibody to be biotinylated at a concentration of 10 mg/ml. After 30-60 min at room temperature, unreacted biotinylation reagent and reaction-by-products are removed by gel filtration on Sephadex G-25 (Pharmacia) equilibrated with 20 mM sodium phosphate, 0.15 M NaCl, pH 7.2. The level of biotin incorporation is determined using the HABA dye (4'-hydroxyazobenzene-2-carboxylic acid) procedure (Green, N.M. Biochem. J. 94, 23c-24c, 1965).

VI.8.4.2. Derivatization of streptavidin with hapten residues

Fluorescein isothiocyanate (FITC; Pierce Chemical Company, Rockford, IL, USA) is dissolved in DMF at a concentration of 2 mg/ml (from light protected). An aliquot of 50-100 µl of this solution is added to each ml of 0.1 M sodium carbonate, pH 9.5, containing streptavidin at a concentration of 2-4 mg/ml. After incubation overnight at 4 °C, excess fluorescein is removed by gel filtration on Sephadex G-25 (Pharmacia) equilibrated with 20 mM sodium phosphate, 0.15 M NaCl, pH 7.2.

VI.9. RELEASE OF ENCAPSULATED REDOX MEDIATORS FROM AFFINITY LIPOSOMES

Method A. Utilizing cholesterol-containing affinity liposomes, the release of encapsulated redox mediators is mediated by the addition of detergent (i.e., Triton X-100 or sodium deoxycholate) or organic solvent. The percentage of detergent or organic solvent required for lysis of cholesterol-containing affinity liposomes is dependent on the percentage of cholesterol in the liposomal bilayer. For example, the integrity of the bilayer of affinity liposomes prepared from a mixture containing phosphatidyl choline, cholesterol, phosphatidyl glycerol, and phosphatidyl ethanolamine derivatized with a reactive residue at a molar ratio of 8: 10: 1: 1, will be stable up to a level of organic solvent addition of about 5%. In contrast, affinity liposomes prepared from a mixture containing only phosphatidyl choline and phosphatidyl glycerol at a molar ratio of 4: 1, are completely disrupted in the presence of 0.4% Triton X-100.

Method B. Utilizing temperature-sensitive, cholesterol-free affinity liposomes, the release of encapsulated redox mediators is mediated by an increase of the ambient temperature (phase-transition release) as described by Magin, R.L. et al. (In: Liposome Technology (G. Gregoriadis, ed.), vol. III., pp. 137-155, CRC Press, Boca Raton, Fl., 1984). Preferred heating rates at passage through T_m are 10 to 15 °C/min.

VI.10. SYNTHESIS OF POLYMERIC CARRIER SYSTEMS FOR AMPLIFIED ASSAY PROCEDURES

Soluble dextran polymers of molecular weight between 10,000 and 50,000 have been used extensively as carriers of proteins and other molecules including the application as a carrier of biotin residues (Brandt, H.M. et al., J. Neurosci. Methods 45, 35, 1992) and hapten molecules (Shi, L.B. et al., Cancer Res. 51, 4192, 1991).

VI.10.1. Activation of dextran polymers

VI.10.1.1. Synthesis of polyaldehyde derivatives of dextran

Dextran of molecular weight between 10,000 and 40,000 is dissolved in a 30 mM aqueous sodium periodate solution (6.42 g $NaIO_4$ in 500 ml deionized water) and stirred overnight at room temperature in the dark. Excess reactant is removed by dialyis against water and the purified polyaldehyde dextran is lyophilized for long-term storage.

The degree of aldehyde formation may be assessed by aldehyde-mediated reduction of Cu^{2+} to Cu^{+} which can be detected using the bicinchoninic acid (BCA) reagent (Pierce Chemical Company, Rockford, IL, USA) as described by Smith, P.K. et al., Anal. Biochem. 150, 76, 1985. The formation of Cu^{+} is in direct proportion to the amount of aldehydes present in the polymer. BCA forms a purple-colored complex with Cu^{+} which can be measured at 562 nm.

VI.10.1.2. Synthesis of polyamine and polyhydrazide derivatives of dextran

For the preparation of polyamine derivatives of dextran, ethylene diamine (or another suitable diamine) is dissolved in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2, at a concentration of 3 M. To avoid pH adjustment of the highly alkaline free-base form of ethylene diamine, the hydrochloride form of ethylene diamine is utilized. For the preparation of polyhydrazide derivatives of dextran, adipic acid dihydrazide (or another suitable dihydrazide compound) is dissolved in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2, at a concentration of 30 mg/ml (heating under a hot water tap is necessary to dissolve the dihydrazide compound completely at this concentration) and the pH is adjusted to 7.2 with HCl. The diamine- or dihydrazide-containing solution is used to dissolve polyaldehyde dextran (prepared as described in section VI.10.1.1.) at a concentration of 25 mg/ml and each ml of the polyaldehyde dextran/diamine (or polyaldehyde dextran/dihydrazide) solution is mixed with 0.2 ml of 1 M sodium cyanoborohydride (in a fume hood). After reaction for at least 6 hrs at room temperature, excess diamine (or dihydrazide) and reaction-by-products are removed by dialysis.

VI.10.1.3. Synthesis of polycarboxymethyl derivatives of dextran

In a fume hood, a solution consisting of 1 M chloroacetic acid in 3 M NaOH is prepared and immediately used to dissolve dextran polymer at a concentration of 40 mg/ml. After reaction for 70 min at room temperature with stirring, the reaction is stopped by adding 4 mg/ml of solid NaH_2PO_4 and adjusting the pH to neutral with 6 M HCl. Excess reactants are removed by dialysis.

VI.10.1.4. Synthesis of lactone derivatives of polycarboxymethyl-dextran

The lactone derivative of polycarboxymethyl-dextran is prepared by refluxing polycarboxymethyl-dextran (section VI.10.1.3.) for 5 hrs in toluene or in other anhydrous solvents as described by Heindel, N.D. et al. (Bioconjugate Chem. 5, 98, 1994). The lactone derivative is highly reactive towards amine-containing molecules.

VI.10.1.5. Synthesis of epoxy-activated derivatives of dextran

*Bis*oxirane compounds are utilized to introduce epoxide functional groups into soluble dextran polymers as described by Böcher, M. et al. (J. Immunol. Meth. 151, 1, 1992). Epoxide functional groups react efficiently with sulfhydryl groups at pH values ranging between 7.5 and 8.5, and with amine nucleophiles at moderate alkaline pH values (typically needing pH values of at least 9). In a fume hood, 1,4-butanediol diglycidyl ether is mixed with an equal part of 0.6 M NaOH containing 2 mg/ml sodium borohydride. With stirring, 5 mg of dextran are added to each ml of the *bis*-epoxide solution. After reaction for 12 hrs at 25 °C, excess reactants are removed by extensive dialysis. For long-term storage, the activated dextran is lyophilized.

VI.10.1.6. Synthesis of polypyridyl disulfide derivatives of dextran

For the preparation of polypyridyl disulfide derivatives of dextran, polyamine-derivatized (or polyhydrazide-derivatized) dextran polymers (prepared as described in section VI.10.1.2.) are reacted with the heterobifunctional cross-linking reagent SPDP (Pierce Chemical Company, Rockford, IL, USA). SPDP is dissolved at a concentration of 6.2 mg/ml in DMF (makes a 20 mM stock solution) and 100 µl of this solution are added to each ml of 50 mM sodium phosphate, pH 7.5, containing the polyamine-derivatized (or polyhydrazide-derivatized) dextran polymer at a concentration of 20 mg/ml. After reaction for 3 hour at room temperature, excess reagents are removed by dialysis.The degree of pyridyl disulfide derivatization is determined by monitoring the release of the pyridine-2-thione leaving groups (characteristic absorbance at 343 nm; $\varepsilon = 8.08 \times 10^3\ M^{-1}\ cm^{-1}$) after incubation of an aliquot for 15 min at room temperature in the presence of 20 mM dithiothreitol

VI.10.1.7. Synthesis of polythioester derivatives of dextran

For the preparation of polythioester derivatives of dextran, polyamine-derivatized (or polyhydrazide-derivatized) dextran polymers (prepared as described in section VI.10.1.2.) are reacted with the heterobifunctional cross-linking reagent SATA (Pierce Chemical Company, Rockford, IL, USA). SATA is dissolved at a concentration of 8 mg/ml in DMF and 100 µl of this solution are added to each ml of 50 mM sodium phosphate, pH 7.5, containing the polyamine-derivatized (or polyhydrazide-derivatized) dextran polymer at a concentration of 20 mg/ml. After reaction for 3 hour at room temperature, excess reagents are removed by dialysis.

To deprotect the thioacetyl group, 100 µl of 500 mM hydroxylamine hydrochloride, 25 mM EDTA, pH 7.5, are added to each ml of SATA-derivatized dextran polymers and reacted for 2 hours at 37 °C. The sulfhydryl-containing dextran polymers are used immediately for further reaction with sulfhydryl-reactive molecules.

VI.10.1.8. Synthesis of polyiodoacetyl derivatives of dextran

For the preparation of polyiodoacetyl derivatives of dextran, polyamine-derivatized (or polyhydrazide-derivatized) dextran polymers (prepared as described in section VI.10.1.2.) are reacted with iodoacetic acid in the presence of the water-soluble carbodiimide EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; Pierce Chemical Company, Rockford, IL, USA). A solution of 0.1 M MES (4-morpholineethanesulfonic acid), 0.15 M NaCl, pH 4.7, containing iodoacetic acid at a concentration of 10 mM and polyamine-derivatized (or polyhydrazide-derivatized) dextran polymers at a concentration of 20 mg/ml, is added to solid EDC (10 mg per ml of polyamine-derivatized dextran/iodoacetic acid mixture), mixed well, and reacted for 3 hrs at room temperature with stirring. Excess reagents are removed by extensive dialysis.

VI.10.2. Coupling of affinity components to dextran polymers

Dextran polymers suitable for amplified assay procedures contain two types of affinity components. One of the covalently linked affinity components is capable of specifically binding to a specific captured target nucleic acid or its amplicons in a structure restricted manner (e.g., intercalating agents or oligonucleotides). The other covalently linked affinity components are capable of specifically binding multiple affinity liposomes. Suitable affinity systems mediating the binding of affinity liposomes to dextran polymers include hapten / anti-hapten antibody affinity systems, enzyme inhibitor / enzyme affinity systems, and the biotin / (strept)avidin affinity system. For example, affinity liposomes containing surface-attached proteinaceous affinity components (e.g., anti-hapten antibodies, enzyme molecules, or (strept)avidin) may be utilized for the detection of dextran polymers containing the corresponding low molecular weight affinity partner (e.g., hapten molecules, enzyme inhibitors, or biotin residues) and nucleic acid-reactive affinity components (e.g., intercalating agents or oligonucleotides).

VI.10.2.1. Coupling of oligonucleotides and methotrexate to polyaldehyde-dextran

In this example, hydrazide-derivatized methotrexate (a high affinity inhibitor of the enzyme dihydrofolate reductase) and hydrazide-derivatized oligonucleotides

(prepared as described in section VI.1.1.) are coupled to polyaldehyde derivatives of dextran (prepared as described in section VI.10.1.1.).

VI.10.2.1.1. *Derivatization of methotrexate with a hydrazide residue.*

Methotrexate (MTX)-γ-hydrazide is prepared from 4-amino-4-deoxy-N^{10}-methylpteroic acid (APA) obtained by cleavage of MTX with carboxypeptidase G_1 (Martinelli, J.A. et al., J. Med. Chem. 22, 869, 1979), and L-glutamic acid α-*tert*-butyl γ-methyl ester with the aid of the peptide bond forming reagent diethylphosphoro cyanidate (Rosowsky, A. et al. J. Med. Chem. 24, 1450, 1981).

a) Synthesis of L-glutamic acid α-*tert*-butyl γ-methyl ester. A mixture of L-glutamic acid γ-methyl ester (1.29 g, 8 mmol), t-BuOAc (60 ml), and 70% $HClO_4$ (1.26 g, 8.8 mmol) is stirred at room temperature for 3 days, cooled to 0 °C, and extracted with cold 0.5 M HCl. The acidic solution is neutralized with powdered $NaHCO_3$ and extracted with Et_2O. Washing with saturated NaCl, drying, and solvent evaporaation yields the product as semisolid, which is coupled directly to APA.

b) Synthesis of MTX α-*tert*-butyl γ-methyl ester. APA (2.20 g, 6 mmol), diethylphosphoro cyanidate (2.94 g, 18 mmol), and Et_3N (1.2 g, 12 mmol) are dissolved in 200 ml of dry DMF. The mixture is heated to 80 °C for 2 min and cooled back to room temperature before adding a seocnd portion of Et_3N (1.2 g, 12 mmol) and freshly prepared L-glutamic acid α-*tert*-butyl γ-methyl ester (1.30 g, 6 mmol). Heating is resumed at 80 °C for 2 hours, the solvent is removed by rotary evaporation, and the residue is taken up in $CHCl_3$. After washing with 5% $NaHCO_3$ and solvent evaporation, the product is purified by column chromatography on silica gel with 95/5 $CHCl_3$/MeOH as the eluent.

c) Synthesis of MTX α-*tert*-butyl ester γ-hydrazide. Hydrazine hydrate (0.5 ml) in MeO (5 ml) is added to a solution of MTX α-*tert*-butyl γ-methyl ester (0.52 g, 1 mmol) in MeOH (15 ml), and the solution is kept at 4 °C for 3 days. After vacuum evaporation of most of the solvent, $CHCl_3$ is added with just enough MeOH to bring the solid into solution. After extraction with 5% $NaHCO_3$ and evaporation of the organic layer, the product is purified by column chromatography on silica gel (9: 1; $CHCl_3$: MeOH).

d) Synthesis of MTX γ-hydrazide. A solution of MTX α-*tert*-butyl ester γ-hydrazide (1.56 g, 3 mmol) in 1 N HCl (25 ml) is kept at 50 °C for 1 hour, then cooled, and basified to pH > 9 with 5% NaOH. After adjustment to pH ~8 with AcOH and NH_4OH, the solution is freeze-dried. MTX γ-hydrazide is purified by chromatography on a DEAE-cellulose column which is eluted first with 0.5% NH_4HCO_3 and then with 3% NH_4HCO_3.

VI.10.2.1.2. *Derivatization of dextran-polyaldehyde with hydrazide derivatives of methotrexate and oligonucleotides*

The polyaldehyde derivative of dextran is dissolved in 0.1 M sodium bicarbonate, 0.15 M NaCl, pH 8.5, at a concentration of 20 mg/ml and mixed with a two-fold molar excess (over the molar concentration of aldehyde groups) of each MTX γ-hydrazide and 5'-hydrazide-derivatized oligonucleotides. In a fume hood, to each ml of this mixture 0.2 ml of 1 M sodium cyanoborohydride are added and the reaction mixture is incubated for at least 6 hrs at room temperature. To block remaining aldehydes, 0.2 ml of 1 M Tris-HCl, pH 8, is added to each ml of the reaction mixture, and after an additional 2 hrs at room temperature, the dextran-MTX-oligonucleotide conjugates are purified by dialysis or gel filtration using a column of Sephacryl S-200.

VI.10.2.2. Coupling of oligonucleotides and biotin to polythioester-dextran

In this example, pyridyl disulfide-derivatized oligonucleotides (prepared as described in section VI.1.3.) and pyridyl disulfide-containing biotin (biotin-HPDP; N-[6-(biotinamido)hexyl]-3'-(2'-pyridyldithio)propionamide; Pierce Chemical Company, Rockford, IL, USA) are coupled to deprotected polythioester-derivatives of dextran (prepared as described in section VI.10.1.7.).

After deprotection of the acetylthioester groups by treatment with neutral hydroxylamine, the sulfhydryl-containing dextran polymer is used immediately for coupling of the pyridyl disulfide derivatives of biotin and the oligonucleotides. The sulfhydryl-containing dextran polymer dissolved in 50 mM sodium phosphate, 2.5 mM EDTA, 50 mM NH_2OH, pH 7.5 (20 mg sulfhydryl-containing dextran per ml), is mixed with a two-fold molar excess (over sulfhydryl groups on the dextran polymer) of both, biotin-HPDP dissolved in DMS at a concentration of 20 mM, and the pyridyl disulfide-derivatized oligonucleotides dissolved in 50 mM sodium phosphate, pH 7.5. After 3 hrs at room temperature, the dextran-biotin-

oligonucleotide conjugates are purified by dialysis or gel filtration using a column of Sephacryl S-200.

VI.10.2.3. Coupling of oligonucleotides and hapten residues to polythioester-dextran

In this example, vinylsulfone PEG-derivatized oligonucleotides (prepared as described in section VI.1.6.) and vinylsulfone PEG-derivatized fluorescein are coupled to deprotected polythioester-derivatives of dextran (prepared as described in section VI.10.1.7.).

VI.10.2.3.1. *Derivatization of fluorescein-5-thiosemicarbazide with NHS-PEG-VS*

Fluorescein-5-thiosemicarbazide (Molecular Probes, Eugene, OR, USA) dissolved in DMF at a concentration of 10 mM is mixed with an equal volume of DMF containing NHS-PEG-VS (MW 3400) or NHS-PEG-VS (MW 2000; both Shearwater Polymers Europe, Enschede, Netherlands) at a concentration of 10 mM. After reaction at room temperature overnight (protected from light), four volumes of 50 mM sodium phosphate, pH 7.5, are added to the reaction mixture, and the vinylsulfone PEG-fluorescein conjugates are purified by gel filtration on Sephadex G-10 (Pharmacia) equilibrated with 50 mM sodium phosphate, pH 7.5.

VI.10.2.3.2. *Derivatization of sulfhydryl-containing dextran with vinylsulfone -PEG derivatives of fluorescein and oligonucleotides*

After deprotection of the acetylthioester groups by treatment with neutral hydroxylamine, the sulfhydryl-containing dextran polymer is used immediately for coupling of the vinylsulfone derivatives of fluorescein and the oligonucleotides. The sulfhydryl-containing dextran polymer dissolved in 50 mM sodium phosphate, 2.5 mM EDTA, 50 mM NH_2OH, pH 7.5 (20 mg sulfhydryl-containing dextran per ml), is mixed with a two-fold molar excess (over sulfhydryl groups on the dextran polymer) of both, the vinylsulfone PEG-fluorescein conjugate dissolved in 50 mM sodium phosphate, pH 7.5, and the vinylsulfone PEG-derivatized oligonucleotides dissolved in 50 mM sodium phosphate, pH 7.5. After 3 hrs at room temperature, the dextran-fluorescein-oligonucleotide conjugates are purified by dialysis or gel filtration using a column of Sephacryl S-200.

VI.10.2.4. Coupling of proteins to polyaldehyde-dextran

In this example, non-derivatized IgG antibodies with specificity for double- and/or triple-stranded nucleic acids and non-derivatized streptavidin are coupled to polyaldehyde derivatives of dextran.

The polyaldehyde derivative of dextran is dissolved in 0.1 M sodium bicarbonate, 0.15 M NaCl, pH 8.5, at a concentration of 20 mg/ml. To each ml of polyaldehyde-dextran solution 5 mg IgG antibody dissolved in 0.5 ml of 0.1 M sodium bicarbonate, 0.15 M NaCl, pH 8.5, and 5 mg streptavidin dissolved in 0.5 ml of 0.1 M sodium bicarbonate, 0.15 M NaCl, pH 8.5, are added. In a fume hood, to each ml of this mixture 0.2 ml of 1 M sodium cyanoborohydride is added and the reaction mixture is incubated for at least 6 hrs at room temperature. To block remaining aldehydes, 0.2 ml of 1 M Tris-HCl, pH 8, is added to each ml of the reaction mixture, and after an additional 2 hrs at room temperature, the dextran-antibody-streptavidin conjugates are purified by gel filtration using a column of Sephacryl S-200 or S-300.

VI.11. BASIC ASSAY PROCEDURE

Detection of Mycobacterium tuberculosis DNA using oligonucleotide-affinity liposomes

VI.11.1. Assay principle

Single-stranded (ss) oligonucleotides complementary to the 3'-terminus of short fragments from the direct-repeat region of the *Mycobacterium tuberculosis* DNA are covalently immobilized onto silica beads to serve as capture oligonucleotides. Captured *Mycobacterium tuberculosis* DNA is detected with ss oligonucleotide-affinity liposomes containing p-aminophenol. The liposome-attached oligonucleotides are complementary to the 5'-terminus of short fragments from the direct-repeat region of the *Mycobacterium tuberculosis* DNA. Bound liposomes are lysed by the addition of detergent and released p-aminophenol (PAP) is quantified via redox recycling using interdigitated array (IDA) electrodes. The assay is performed as a combination of liquid chromatography with electrochemical detection (LCEC).

VI.11.2. Instrumentation

The analytical system applied for the detection of *Mycobacterium tuberculosis* DNA includes a micromachined flow-through assembly, a multipotentiostat, pumps, valves, and a computer (PC type). The flow is accomplished using a peristaltic pump combined with a 6-way selector valve. The micromachined flow-through assembly consists of three main parts: a hybridization chamber, a flow chamber designed as a channel structure, and the interdigitated electrode array (IDA). The hybridization chamber (volume: 100 µl) contains silica beads with

covalently immobilized capture oligonucleotides and is connected via a two-way selector valve to a flow channel fabricated by double-sided anisotropic etching in silicon wafers. The lateral dimensions of the flow channel corresponds to the active electrode area (1mm x 3mm) and the channel hight is set to 200 µm. The inlet and outlet tubes are pasted in an acrylic holder and arranged on top of the electrode. The microelectrode arrays consist of platinum electrodes fabricated on thermal oxidized silicon wafers by photolithography and the lift-off technique. One chip contains four independent interdigitated electrode pairs and has an area of 8 mm x 8 mm. One of the 70 fingers of each electrode is 1.5 □m wide. Two adjacent fingers are 0.8 µm spaced.

VI.11.3. Assay components

VI.11.3.1. Oligogonucleotides complementary to short fragments from the direct-repeat region of the *Mycobacterium tuberculosis* DNA.

a) ss Capture oligonucleotide (18-mer): The oligonucleotide is complementary to the 3'-terminus of the short fragment from the direct-repeat region of the *Mycobacterium tuberculosis* DNA and includes at the 3'-terminus i) a 2-base thiophosphate thymine-Ts tag for immobilization to tresyl-activated silica beads and ii) a single T-base separating the tag from the sensing oligonucleotide sequence.

3'-TsTT CAG CAG TCT GGG TTT TGG-5'

b) A short fragment (36-mer) from the direct-repeat region of the *Mycobacterium tuberculosis* DNA.

5'-GTC GTC AGA CCC AAA ACC CCG AGA GGG GAC GGA AAC-3'

c) ss Liposome-attached oligonucleotide (18-mer): The oligonucleotide is complementary to the 5'-terminus of the short fragment from the direct-repeat region of the *Mycobacterium tuberculosis* DNA and includes at the 5'-terminus a pyridyl disulfide residue for covalent coupling to sulfhydryl-derivatized affinity liposomes.

3'-GGC TCT CCC CTG CCT TTG-5'-pyridyl disulfide

Derivatization of the 18-mer oligonucleotide with a pyridyl disulfide residue at the 5'-terminus is performed in two steps as described in VI.1.1. and VI.1.3.

VI.11.3.2. Immobilized capture oligonucleotides. The thiophosphate thymine-containing capture oligonucleotides are immobilized onto tresyl-activated silica beads as described in VI.1.7.6.

VI.11.3.3. Oligonucleotide-affinity liposomes containing p-aminophenol. First, sulfhydryl-derivatized affinity liposomes are prepared by the injection method (Biochim. Biophys. Acta 298, 1015, 1973) from a lipid mixture of dimyristoylphosphatidylcholine, cholesterol, dicetylphosphate at a molar ratio of 5:4:1, and phosphatidylethanolamine derivatized with succinimidyl acetylthiopropionate (PE-SATP) at a concentration of 0.5 mol% of total lipid. PE-SATP is prepared as described in VI.5.5. To prepare liposomes, 2 µmol of stock lipid mixture in chloroform is evaporated under a stream of nitrogen and then placed in a vacuum desiccator overnight. The lipid is resolubilized in 50 µl of dry isopropanol and injected with a syringe into 1 ml of 10 mM HEPES, pH7.4, containing 50 mM p-aminophenol, which is being mixed by vortex. Liposomes of uniform size are formed spontaneously by this method. After removal of unencapsulated p-aminophenol by gel filtration, liposomes are suspended in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.5, at a concentration of 5 mg/ml and mixed with hydroxylamine hydrochloride as described in VI.8.1.1. After 2 hrs at room temperature, the deacetylated liposomes are purified by gel filtration and used immediately for coupling of pyridyl disulfide-derivatized oligonucleotides to the surface-attached sulfhydryl groups (compare VI.8.1.2.).

VI.11.4. Assay procedure

First, the analytical system is washed with several volumes of 20 mM sodium phosphate, 0.1 M NaCl, pH 7.0. Thereafter, the valve connecting the hybridization chamber with the flow chamber is closed and the sample (50 µl) containing the short fragment (36-mer) from the direct-repeat region of the *Mycobacterium tuberculosis* DNA is applied to the hybridization chamber. After an incubation of 1 hr at room temperature, the hybridization chamber is washed with several volumes of 20 mM sodium phosphate, 0.1 M NaCl, pH 7.0, captured *Mycobacterium tuberculosis* DNA is detected by the addition of 50 µl oligonucleotide-affinity liposomes (approximately 500 ng of liposome-attached oligonucleotide ml^{-1}). After an incubation for an additional 1 hr at room temperature, the hybridization chamber is washed again with several volumes of 20 mM sodium phosphate, 0.1 M NaCl, pH 7.0. Finally, the valve connecting the hybridization chamber with the flow chamber is opened and bound oligonucleotide-affinity liposomes are lysed by the addition of 100 µl of 10 mM sodium phosphate, pH 7.0, containing 0.01% Triton X-100. Released p-aminophenol (PAP) is detected by redox recycling. PAP is oxidized to quinoneimine at the anode (+250 mV) yielding an oxidation current, and the quinoneimine is reduced to PAP at the cathode (-50 mV).

VI.11.5. Detection sensitivity

Using redox recyling for the detection of PAP, the detection limit is in the range of 50 nM corresponding to 2.5 pmol per 50 µl. Since 10^4 - 10^5 p-aminophenol molecules are released from a single bound oligonucleotide-affinity liposome, the described assay configuration allows the detection of low nanogram quantities per ml of the short fragment (36-mer) from the direct-repeat region of the *Mycobacterium tuberculosis* DNA (1 ng corresponds to 85 fmol).

VI.12. AMPLIFIED ASSAY PROCEDURE

Detection of Detection of Mycobacterium tuberculosis DNA using preformed complexes of biotinylated oligonucleotides, streptavidin, and affinity liposomes

VI.12.1. Assay principle

Single-stranded (ss) oligonucleotides complementary to the 3'-terminus of short fragments from the direct-repeat region of the *Mycobacterium tuberculosis* DNA are covalently immobilized onto silica beads to serve as capture oligonucleotides. Captured *Mycobacterium tuberculosis* DNA is detected with preformed complexes of biotinylated oligonucleotides, streptavidin, and affinity liposomes containing p-aminophenol. The biotinylated oligonucleotides are complementary to the 5'-terminus of short fragments from the direct-repeat region of the *Mycobacterium tuberculosis* DNA. Bound complexes of biotinylated oligonucleotides, streptavidin, and affinity liposomes are lysed by the addition of detergent and released p-aminophenol (PAP) is quantified via redox recycling using interdigitated array (IDA) electrodes. The assay is performed as a combination of liquid chromatography with electrochemical detection (LCEC).

VI.12.2. Instrumentation

The analytical system applied for the detection of *Mycobacterium tuberculosis* DNA using the amplified assay procedure is the same as described in VI.11.2.

VI.12.3. Assay components

VI.12.3.1. Oligogonucleotides complementary to short fragments from the direct-repeat region of the *Mycobacterium tuberculosis* DNA.

a) ss Capture oligonucleotide (18-mer): The oligonucleotide is complementary to the 3'-terminus of the short fragment from the direct-repeat region of the *Mycobacterium tuberculosis* DNA and includes at the 3'-terminus i) a 2-base

thiophosphate thymine-Ts tag for immobilization to tresyl-activated silica beads and ii) a single T-base separating the tag from the sensing oligonucleotide sequence.

3'-TsTsT CAG CAG TCT GGG TTT TGG-5'

b) A short fragment (36-mer) from the direct-repeat region of the *Mycobacterium tuberculosis* DNA.

5'-GTC GTC AGA CCC AAA ACC CCG AGA GGG GAC GGA AAC-3'

c) ss Biotinylated oligonucleotide (18-mer): The oligonucleotide is complementary to the 5'-terminus of the short fragment from the direct-repeat region of the *Mycobacterium tuberculosis* DNA and includes at the 5'-terminus a biotin residue.

3'-GGC TCT CCC CTG CCT TTG-5'-*biotin residue*

5'-Biotinylated oligonucleotides are synthesized by derivatization of 5'-amine derivativatives (prepared as described in VI.4.1.) with succinimidyl-6-(biotinamido)hexanoate (NHS-LC-biotin) as described in Bioconjugate Techniques (G.T. Hermanson, ed.) p. 658, Academic Press, San Diego, CA, 1996.

VI.12.3.2. Immobilized capture oligonucleotides. The thiophosphate thymine-containing capture oligonucleotides are immobilized onto tresyl-activated silica beads as described in VI.1.7.6.

VI.12.3.3. Preformed complexes of biotinylated oligonucleotides, streptavidin, and affinity liposomes containing p-aminophenol. Biotinylated affinity liposomes containing p-aminophenol are prepared by the injection method (Biochim. Biophys. Acta 298, 1015, 1973) from a lipid mixture of dimyristoylphosphatidylcholine, cholesterol, dicetylphosphate at a molar ratio of 5:4:1, and N-biotinyldipalmitoyl-L-↤-phosphatidylethanolamine (B-PE; Molecular Probes, Junction City, OR, USA) at a concentration of 0.1mol% of total lipid. . To prepare liposomes, 2 µmol of stock lipid mixture in chloroform is evaporated under a stream of nitrogen and then placed in a vacuum desiccator overnight. The lipid is resolubilized in 50 µl of dry isopropanol and injected with a syringe into 1 ml of 10 mM HEPES, pH 7.4, containing 50 mM p-aminophenol, which is being mixed by vortex. Liposomes of uniform size are formed spontaneously by this method. After removal of unencapsulated p-aminophenol by gel filtration, liposomes are continuously stirred in 20 mM Tris, 0.15 M NaCl, 0.01 % (v/v) NaN_3, pH 7.4, at a concentration of 0.5 nmol total lipid in 2.5 ml buffer. Streptavidin is quantified using an extinction coefficient of 15.4 cm^{-1} for a 1% (w/v) solution at 281 nm. Preformed complexes of biotinylated oligonucleotides, streptavidin, and biotinylated affinity liposomes are prepared by mixing biotinylated affinity

liposomes containing 2 nmol of N-biotinyldipalmitoyl-L-↔-phosphatidylethanolamine (B-PE) at a level of 0.1 mol% of total lipid with 0.4 nmol of streptavidin (B-PE: streptavidin ratio = 5) for 2 min, followed by 2 nmol of biotinylated oligonucleotide (biotinylated oligonucleotide: strepavidin ratio = 5) for 10 min.

VI.12.4. Assay procedure

First, the analytical system is washed with several volumes of 20 mM sodium phosphate, 0.1 M NaCl, pH 7.0. Therafter, the valve connecting the hybridization chamber with the flow chamber is closed and the sample (50 µl) containing the short fragment (36-mer) from the direct-repeat region of the *Mycobacterium tuberculosis* DNA is applied to the hybridization chamber. After an incubation of 1 hr at room temperature, the hybridization chamber is washed with several volumes of of 20 mM sodium phosphate, 0.1 M NaCl, pH 7.0, captured *Mycobacterium tuberculosis* DNA is detected by the addition of 50 µl preformed complexes of biotinylated oligonucleotides, streptavidin, and affinity liposomes containing p-aminophenol (approximately 500 ng of biotinylated oligonucleotides ml^{-1}). After an incubation for an additional 1 hr at room temperature, the hybridization chamber is washed again with several volumes of 20 mM sodium phosphate, 0.1 M NaCl, pH 7.0. Finally, the valve connecting the hybridization chamber with the flow chamber is opened and bound complexes of biotinylated oligonucleotides, streptavidin, and affinity liposomes are lysed by the addition of 100 µl of 10 mM sodium phosphate, pH 7.0, containing 0.01% Triton X-100. Released p-aminophenol (PAP) is detected by redox recycling. PAP is oxidized to quinoneimine at the anode (+250 mV) yielding an oxidation current, and the quinoneimine is reduced to PAP at the cathode (-50 mV).

VI.12.5. Detection sensitivity

As compared to the detection sensitivity of the basic assay procedure (VI.11.5), the use of preformed complexes of biotinylated oligonucleotides, streptavidin, and affinity liposomes increases the sensitivity by approximately one order of magnitude.

Claims:

1. A reporter system for detecting an nucleic acid containing analyte present in a liquid, comprising the following components:

a) capture oligonucleotides immobilized on a solid support and capable of specifically binding said analyte;

b) affinity systems containing encapsulated electrochemically detectable reporter molecules, selected from

(b1) affinity liposomes containing encapsulated electrochemically detectable reporter molecules and comprising at least one surface-attached affinity component capable of specifically binding to said analyte and/or said capture olligonucleotides in a condition where analyte and capture oligonucleotides are bound to each other, but not to free capture oligonucleotides,

(b2) (i) polymeric carrier molecules containing at least one covalently linked affinity component capable of specifically binding to said analyte and/or said capture oligonucleotides in a condition where analyte and capture oligonucleotides are bound to each other, but not to free capture oligonucleotides, and at least one covalently linked affinity component capable of specifically binding to affinity liposomes (ii), plus (ii) said affinity liposomes containing encapsulated electrochemically detectable reporter molecules and comprising at least one surface-attached affinity component capable of specifically binding to said polymeric carrier molecules;

(b3) complexes of affinity liposomes, the single liposomes containing encapsulated electrochemically detectable reporter molecules and comprising at least one surface-attached affinity component capable of specifically binding to said analyte and/or said capture oligonucleotides in a condition where analyte and capture oligonucleotides are bound to each other, but not to free capture oligonucleotides;

(b4) (i) polymeric carrier molecules containing at least one covalently linked affinity component capable of specifically binding to said analyte and/or said capture oligonucleotides in a condition where analyte and capture oligonucleotides are bound to each other, but not to free capture

oligonucleotides, and at least one covalently linked affinity component capable of specifically binding to affinity liposomes of complexes of affinity liposomes (ii), plus (ii) said complexes of affinity liposomes being defined as under (b3); and

c) an electrochemical sensor.

2. The reporter system of claim 1, wherein the liposome-encapsulated electrochemically detectable reporter molecules are selected from redox mediators.

3. The reporter system of claim 2, wherein the liposome-encapsulated electrochemically detectable reporter molecules are selected from the group consisting of aromatic redox mediators and organic and inorganic metal complexes containing osmium, ruthenium, iron, copper, and chromium.

4. The reporter system of claim 3, wherein the aromatic redox mediators are selected from the group consisting of p-aminophenol and derivatives thereof, catechol and derivatives thereof, dopamine and derivatives thereof, methoxytyraminin and derivatives thereof, aromatic compounds with more than one aromatic ring structure including anthracene derivatives, and heterocyclic aromatic compounds including serotonin, hydroxyindol acetic acid, and derivatives thereof.

5. The reporter system of claim 3, wherein the organic metal complexes are selected from the group consisting of metal complexes containing the metal complexed by aromatic and heterocyclic aromatic compounds, the side chains of said compounds being derivatized with residues preferably selected from carboxyl groups, halogens, aminoethyl groups, and pyridine derivatives.

6. The reporter system of claim 1, wherein the or at least one of the surface-attached affinity component(s) of the affinity liposomes capable of specifically binding to captured analyte and/or capture oligonucleotides according to (b1) is selected from the group consisting of single-stranded ribo and deoxyribo nucleic acids, single-stranded ribo and deoxyribo oligonucleotides, 'preorganized' oligonucleotide structures including peptide nucleic acid (PNA) analogs, intercalating agents, intercalating agents conjugated to single-stranded oligonucleotides or nucleic acids, immunoglobulins or fragments of immunoglobulins with specificity for double- and/or triple-stranded nucleic

acids, and non-immunoglobulin proteins capable of specifically binding to double-stranded nucleic acids.

7. The reporter system of claim 6, wherein the said surface-attached affinity component(s) is/are attached to the surface of the affinity liposomes via spacer molecules including oligoalkyl glycol derivatives and monomers or oligomers of 6-aminoalkyl acid derivatives.

8. The reporter system of claim 6, wherein the said surface-attached affinity component(s) is/are an intercalating agent selected from acridines and derivatives thereof, actinomycin D and derivatives thereof, anthracyclines and derivatives thereof, cyanine dyes and derivatives thereof, hydroxystilbamidine and derivatives thereof, imidazoles and derivatives thereof, indoles and derivatives thereof, phenanthridines and derivatives thereof, and psoralens and derivatives thereof.

9. The reporter system of claim 1, wherein the electrochemical sensor consists of a closely spaced array of thin film nobel metal electrodes for voltammetric or amperometric quantification of electrochemical reporter molecules via redox reactions.

10. The reporter system of claim 9, wherein the said electrodes are spaced apart from each other with a distance between 5 and 5000 nm and the anodes and cathodes have a width between 20 and about 800 nm.

11. The reporter system of claim 1, wherein the polymeric carrier molecules (i) according to (b2) are selected from the group consisting of derivatives of synthetic or natural polymers including polysaccharides, polyamino acids, polyvinyl alcohols, polyvinylpyrrolidinones, polyacrylic acids, polyurethanes, polyphosphazenes, and copolymers of such polymers.

12. The reporter system of claim 1, wherein the covalently linked affinity components of the polymeric carrier molecules (i) according to (b2) capable of specifically binding affinity liposomes are selected from the group consisting of haptens, anti-hapten antibodies, enzyme inhibitors, inhibitor-binding enzymes, biotin, avidin, and streptavidin.

13. The reporter system of claim 1, wherein the surface-attached affinity component(s) of the said affinity liposomes (ii) according to (b2) capable of specifically binding to polymeric carrier molecules are selected from the group consisting of haptens, anti-hapten antibodies, enzyme inhibitors, inhibitor-binding enzymes, biotin, avidin, and streptavidin.

14. The reporter system of claim 1, wherein the complexes of affinity liposomes according to (b3) are formed from affinity liposomes containing two types (I and II) of surface-attached affinity components, the type I surface-attached affinity components being capable of specifically binding to said analyte and/or said capture oligonucleotides in a condition where analyte and capture oligonucleotides are bound to each other, but not to free capture oligonucleotides, and preferably being defined as in claim 6, and the type II surface-attached affinity components being capable of mediating the complexation of affinity liposomes via bridging molecules which have at least two binding sites for type II affinity components.

15. The reporter system of claim 14, wherein the surface-attached type II affinity components capable of mediating the complexation of affinity liposomes via bridging molecules are selected from the group consisting of haptens, enzyme inhibitors, and biotin.

16. The reporter system of claim 14, wherein the bridging molecules are selected from molecules with more than one binding site, preferably from the group consisting of a) bi- or oligovalent anti-hapten antibodies, fragments thereof, conjugates thereof, and fusion constructs thereof; b) enzymes, enzyme conjugates, and fusion constructs thereof; and c) avidin and streptavidin.

17. The reporter system of claim 1, wherein the complexes of affinity liposomes according to (b3) are formed (i) from affinity liposomes containing two types (I and II) of surface-attached affinity components, the type I surface-attached affinity components capable of specifically binding to said analyte and/or said capture oligonucleotides in a condition where analyte and capture oligonucleotides are bound to each other, but not to free capture oligonucleotides, and the type II surface-attached affinity components being capable of mediating the complexation of affinity liposomes via polymeric carrier molecules containing covalently linked affinity components providing at

least two binding sites for type II affinity components of said affinity liposomes, and (ii) from the said polymeric carrier molecules.

18. The reporter system of claim 17, wherein the surface-attached type II affinity components capable of mediating the complexation of affinity liposomes via polymeric carrier molecules are selected from a group including haptens, anti-hapten antibodies, enzyme inhibitors, inhibitor-binding enzymes, biotin, avidin, and streptavidin.

19. The reporter system of claim 17, wherein the polymeric carrier molecules are selected from the group consisting of derivatives of synthetic or natural polymers including polysaccharides, polyamino acids, polyvinyl alcohols, polyvinylpyrrolidinones, polyacrylic acids, polyurethanes, polyphosphazenes, and copolymers of such polymers.

20. The reporter liposomes of claim 17, wherein the covalently linked affinity components of the said polymeric carrier molecules (ii) are selected from the group consisting of haptens, anti-hapten antibodies, enzyme inhibitors, inhibitor-binding enzymes, biotin, avidin, and streptavidin.

21. The reporter system of claim 1, wherein the complexes of affinity liposomes according to (b3) are formed from two types (I and II) of affinity liposomes, type I affinity liposomes containing at least two surface-attached affinity components capable of specifically binding to a specific captured oligonucleotide, and type II affinity liposomes containing at least two surface-attached affinity components capable of specifically binding to type I affinity liposomes.

22. The reporter system of claim 21, wherein the affinity components of type I affinity liposomes are selected from single-stranded ribo and deoxyribo oligonucleotides, and 'preorganized' oligonucleotide structures including peptide nucleic acid (PNA) analogs, capable of forming specific helical complexes with specific target nucleic acids or their amplicons, and the affinity components of type II affinity liposomes are selected from single-stranded ribo and deoxyribo oligonucleotides, 'preorganized' oligonucleotide structures including peptide nucleic acid (PNA) analogs, capable of forming specific helical complexes with the oligonucleotides and 'preorganized' oligonucleotide structures of type I affinity liposomes.

23. The reporter system of claim 1, wherein the complexes of affinity liposomes (ii) according to (b4) are formed from affinity liposomes containing two types (I and II) of surface-attached affinity components, the surface-attached type I affinity components being capable of specifically binding to polymeric carrier molecules and preferably being selected from the group consisting of haptens, anti-hapten antibodies, enzyme inhibitors, inhibitor binding enzymes, biotin, avidin, and streptavidin, and the surface-attached type II affinity components being capable of mediating the complexation of affinity liposomes via bridging molecules and preferably being selected from the group consisting of hapatens, enzyme inhibitors, and biotin, the bridging molecules being selected from molecules with more than one binding site, preferably from the group consisting of (a) bi- or oligovalent anti-hapten antibodies, fragments thereof, conjugates thereof, and fusion constructs thereof; b) inhibitor-binding enzymes, conjugates thereof, and fusion constructs thereof; and c) avidin and streptavidin, and providing at least two binding sites for type II affinity components.

24. The reporter system of claim 1, wherein the complexes of affinity liposomes (ii) according to (b4) are formed from affinity liposomes containing two types (I and II) of the surface-attached affinity components, the surface-attached type I affinity components being capable of specifically binding to polymeric carrier molecules and preferably being selected from the group consisting of haptens, anti-hapten antibodies, enzyme inhibitors, inhibitor-binding enzymes, biotin, avidin, and streptavidin, and the surface-attached type II affinity components being preferably selected from the group consisting of haptens, antihapten antibodies, enzyme inhibitors, inhibitor-binding enzymes, biotin, avidin, and streptavidin and being capable of mediating the complexation of affinity liposomes via polymeric carrier molecules containing affinity components preferably being selected from the group consisting of haptens, antihapten antibodies, enzyme inhibitors, inhibitor-binding enzymes, biotin, avidin, and streptavidin, and providing the one or more binding sites for the said type II affinity components.

25. A method for detecting a nucleic acid containing analyte in a liquid sample, comprising the steps of:

a) providing

(i) capture oligonucleotides immobilized on a solid support and capable of

specifically binding said analyte;
(ii) affinity systems comprising affinity liposomes which contain encapsulated electrochemically detectable reporter molecules, the affinity liposomes or other parts of the affinity systems being capable of specifically binding to said analyte and/or said capture oligonucleotides in a condition where analyte and capture oligonucleotides are bound to each other, but not to free capture oligonucleotides, and
(iii) an electrochemical sensor,

b) contacting the sample with the immobilized capture oligonucleotids,
c) adding said affinity systems containing electrochemically detectable reporter molecules,
d) removing unbound affinity liposomes,
e) releasing encapsulated electrochemically detectable reporter molecules from the interior of said affinity liposomes, and
f) measuring the reporter molecules released using the electrochemical sensor.

26. The method of claim 25, wherein the electrochemically detectable reporter molecules are released from the interior of the liposomes by increasing the ambient temperature or by adding a liposome-lysing agent.

27. The method of claim 25, wherein the electrochemical sensor consists of a closely spaced array of thin film nobel metal electrodes for voltammetric or amperometric quantification of electrochemical reporter molecules via redox reactions.

28. The method of claim 25, wherein the capture oligonucleotides immobilized on a solid support are selected from single-stranded oligonucleotides having a sequence complementary to that of the nucleic acid sequence of the analyte to be detected, or to a selected part thereof, and the affinity liposomes are selected from the group of affinity liposomes having surface-attached intercalating agents or antibodies, both being capable of specifically binding to double-stranded nucleic acids.

29. The method of claim 25, wherein the capture oligonucleotides immobilized on a solid suport are selected from single-stranded oligonucleotides having a sequence complementary to a first segment of the nucleic acid sequence of the analyte to be detected and the affinity liposomes are selected from the

group of affinity liposomes having surface-attached single-stranded oligonucleotides, said oligonucleotides comprising a sequence complementary to a second segment of the nucleic acid sequence of the analyte to be detected, or having surface-attached single-stranded oligonucleotide-intercalating agent conjugates being capable of specifically binding to double-stranded nucleic acids.

30. The method of claim 25, additionally comprising the steps of

(g) adding polymeric carrier molecules capable of specifically binding to said analyte and/or said capture oligonucleotides in a condition where analyte and capture oligonucleotides are bound to each other, but not to free capture oligonucleotides, the polymeric carrier molecules at least partly being the said other parts of the affinity systems, and

(h) removing unbound polymeric carrier molecules,

before step (c) is performed, and wherein the affinity liposomes added in step (c) are capable of specifically binding to the said analyte-bound polymeric carrier molecules.

31. The method of claim 30, wherein the capture oligonucleotides are selected from single-stranded nucleic acids or oligonucleotides with a sequence complementary to that of the analyte to be detected or a selected part thereof and the polymeric carrier molecules added according to step (g) contain covalently linked intercalating agents or covalently linked antibodies, both being capable of specifially binding to double-stranded nucleic acids.

32. The method of claim 30, wherein the capture oligonucleotides are selected from single-stranded nucleic acids or oligonucleotides with a sequence complementary to a first segment of the sequence of the analyte to be detected and the polymeric carrier molecules added according to step (g) contain covalently linked oligonucleotides or covalently linked oligonucleotide-intercalating agent conjugates, said oligonucleotides having a sequence complementary to a second segment of the nucleic acid sequence of the analyte to be detected, and said conjugated intercalating agents being capable of specifically binding to double stranded nucleic acids.

33. The method of claim 25, wherein the affinity systems containing electrochemically detectable reporter molecules added in step (c) are preformed complexes of said affinity liposomes.

34. The method of claim 33, wherein the capture oligonucleotides are selected from single-stranded nucleic acids or oligonucleotides with a sequence complementary of that of the analyte to be detected or a selected part thereof, and the preformed complexes of affinity liposomes are selected from the group of complexes containing affinity liposomes having surface-attached intercalating agents or surface-attached antibodies, both being capable of specifically binding to double-stranded nucleic acids.

35. The method of claim 33, wherein the capture oligonucleotides are selected from single-stranded nucleic acids or oligonucleotides with a sequence complementary to a first segment of the sequence of the analyte to be detected, and the preformed complexes of affinity liposomes are selected from the group of complexes containing affinity liposomes having surface-attached single-stranded oligonucleotides comprising a sequence complementary to a second segment of the sequence of the analyte to be detected or having surface-attached single-stranded oligonucleotide-intercalating agent conjugates being capable of specifically binding to double-stranded nucleic acids.

36. The method of of claim 25, comprising the steps of :

(g) adding polymeric carrier molecules capable of specifically binding to said analyte and/or said capture oligonucleotides in a condition where analyte and capture oligonucleotides are bound to each other, but not to free capture oligonucleotides, the polymeric carrier molecules at least partly being the said other parts of the affinity systems, and

(h) removing unbound polymeric carrier molecules,

before step (c) is performed, and wherein the affinity systems added in step (c) are preformed complexes of said affinity complexes capable of specifically binding to the said analyte-bound polymeric carrier molecules.

37. The method of claim 36, wherein the capture oligonucleotides are selected from single-stranded nucleic acids or oligonucleotides with a sequence complementary to that of the analyte to be detected or a selected part thereof, and the polymeric carrier molecules added according to step (g) are capable of specifically binding to captured analyte via covalently linked intercalating agents or covalently linked antibodies both being capable of specifially binding to double-stranded nucleic acids.

38. The method of claim 36, wherein the capture oligonucleotides are selected from single-stranded nucleic acids or oligonucleotides with a sequence complementary to a first segment of the sequence of the analyte to be detected and the polymeric carrier molecules added according to step (g) contain covalently linked single-stranded oligonucleotides or covalently linked single-stranded oligonucleotide-intercalating agent conjugates, said oligonucleotides having a sequence complementary to a second segment of the nucleic acid sequence of the analyte to be detected, and said conjugated intercalating agents being capable of specifically binding to double stranded nucleic acids, respectively.

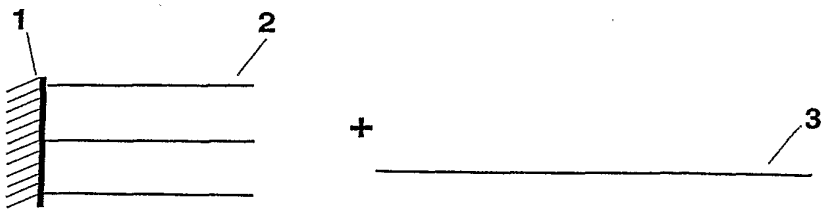


Fig.1a

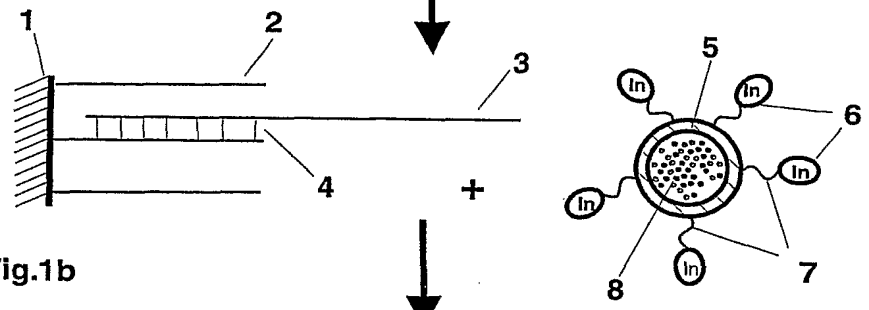


Fig.1b

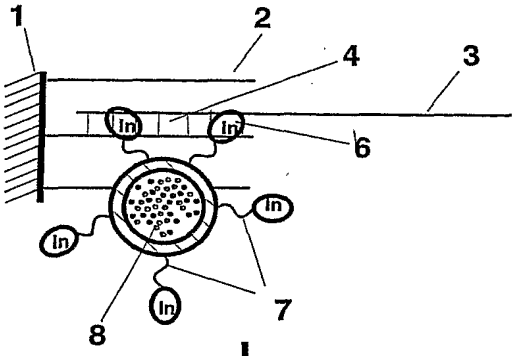


Fig.1c

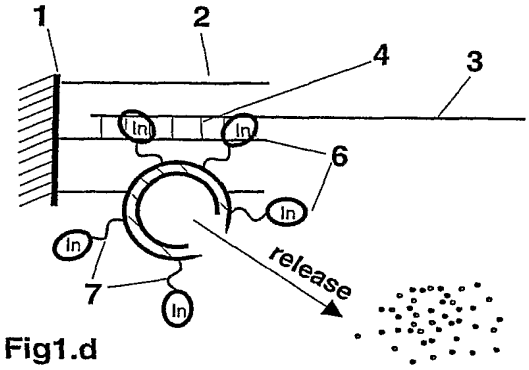


Fig1.d

Fig.1

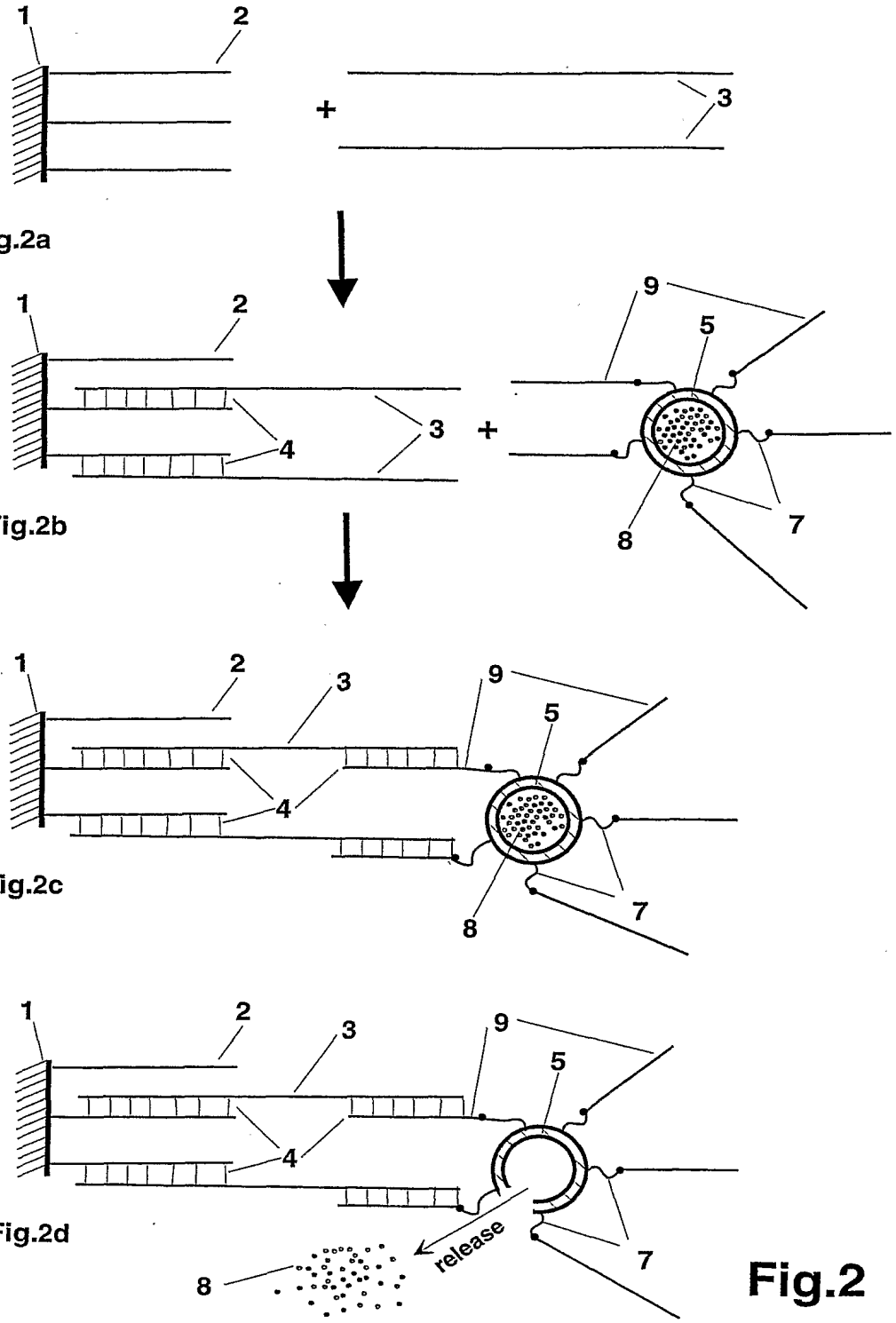

1
2
3
+
Fig.2a
1
2
4
3
+
9
5
8
7
Fig.2b
1
2
3
9
5
4
8
7
Fig.2c
1
2
3
9
5
4
release
8
7
Fig.2d

Fig.2

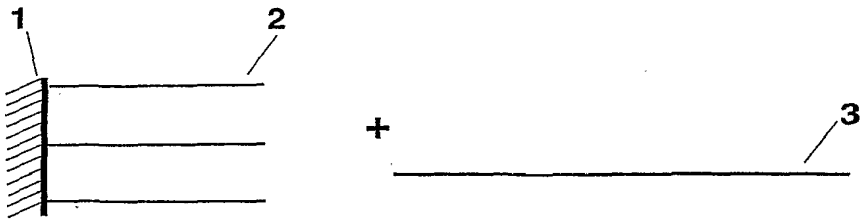


Fig.3a

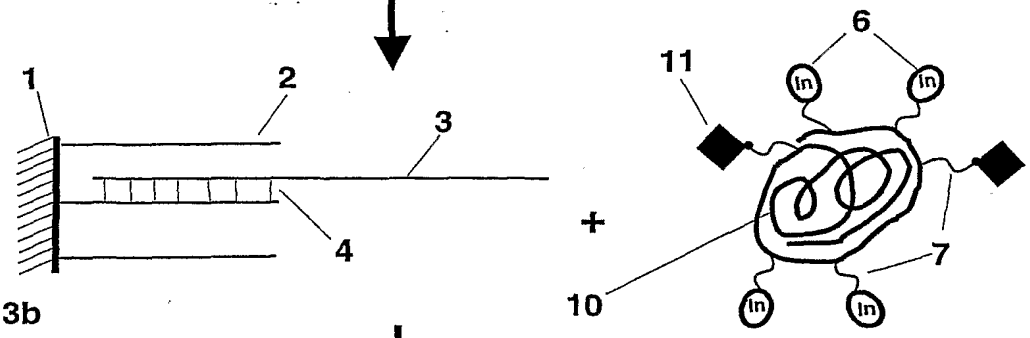


Fig.3b

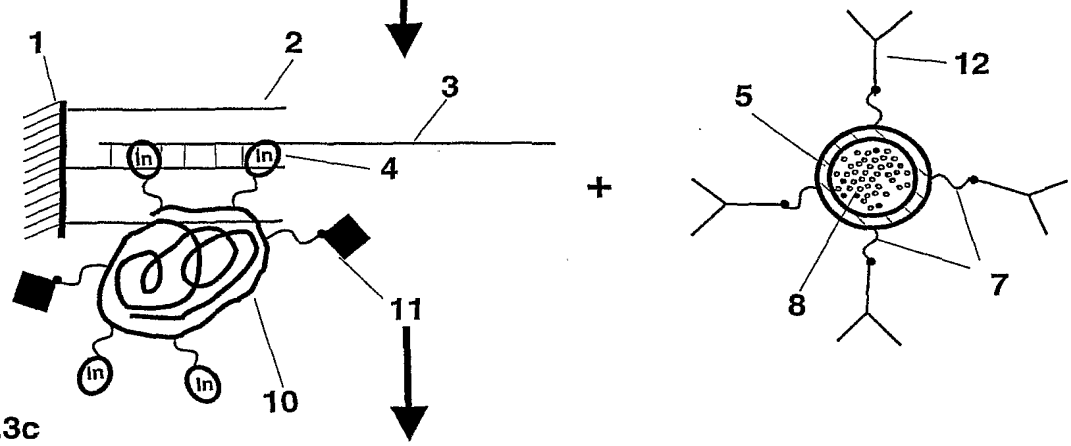


Fig.3c

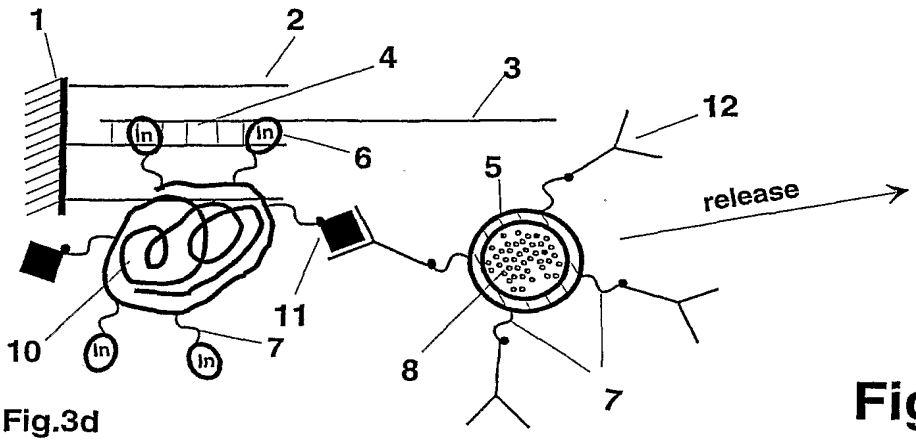
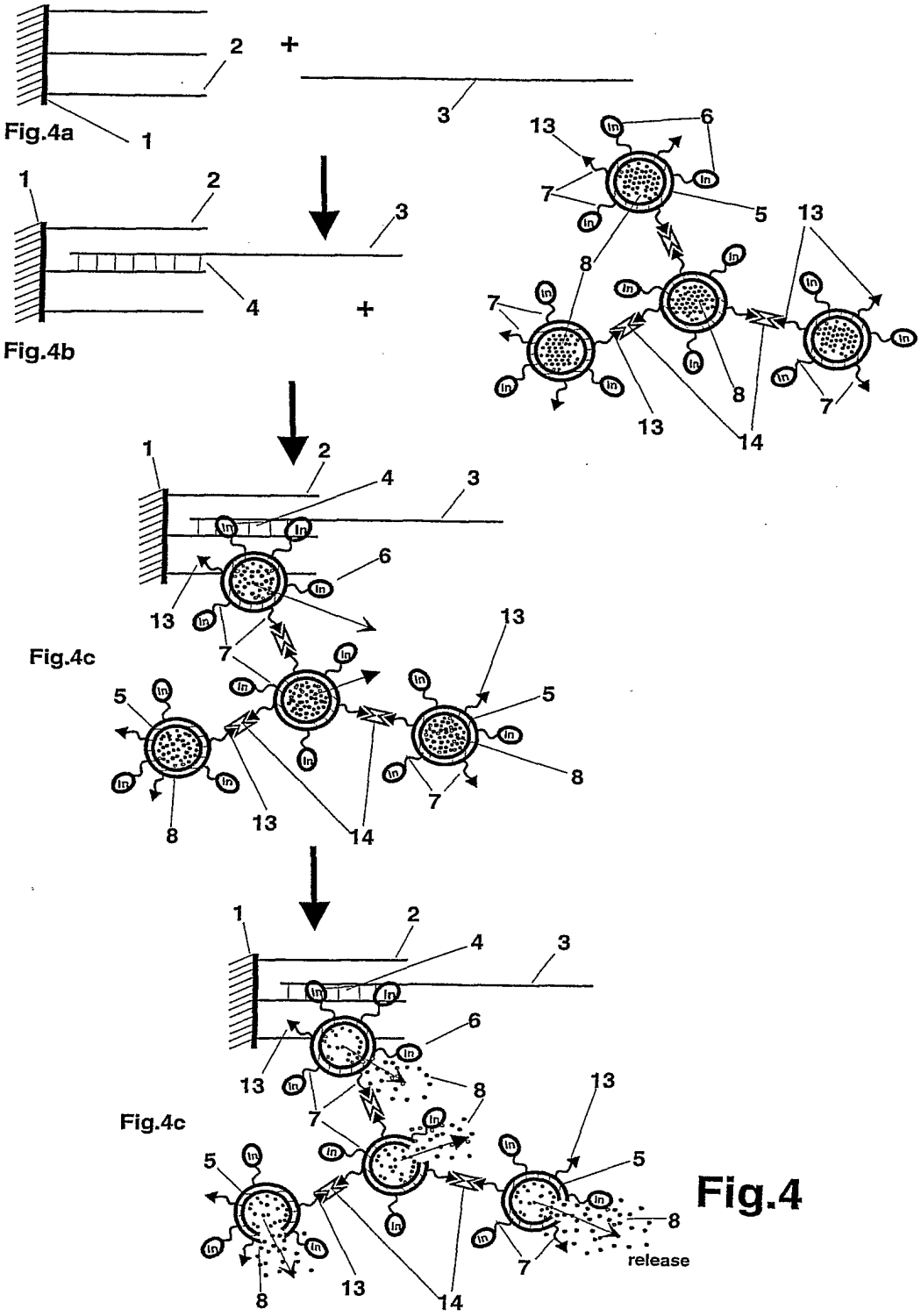


Fig.3d

Fig.3


2
+
3
1
Fig.4a
1
2
3
4
+
Fig.4b
13
6
7
5
8
14
release
Fig.4c
Fig.4c
Fig.4